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Adaptive variation in cold hardiness among aspen (Populus tremuloides) provenances

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ADAPTIVE VARIATION IN COLD HARDINESS AMONG ASPEN (*Populus tremuloides*) PROVENANCES

By

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ABSTRACT

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The relative efficiencies of using different methods to detect genetic variation in cold hardiness were evaluated, and the adaptive variation among aspen provenances was further studied. This thesis is organized as three parts. The first part is a comparison of chlorophyll fluorescence, electrical conductivity and cambium visual scoring to detect genetic variation in cold hardiness of twelve aspen provenances from northwestern Ontario. Comparison of results indicated that rank of provenances in cold hardiness by three methods was similar, but EC is more sensitive to detect variation level among populations. All methods can be a good indicator of adaptive variation, although the adaptive mechanism each method detected may be different. The second part is to evaluate adaptive variation in cold hardiness among 20 aspen provenances from northwestern Ontario using electrical conductivity. Significant variation among provenances was detected, and the variance expressed among provenances was higher than 70%. Principal component analysis summarized 14 variables into three principal components representing different phases of development. PC-1 mainly represented cold hardiness development after late September, while PC-2 and PC-3 mainly explained the cold hardiness in early-mid September. Multiple regression analysis of each PC against modeled climatic variables unique to each seed source indicated that growing season length and precipitation in August were the best predictors of PC-1, while temperatures were always the best predictors of PC-2. The third part of this thesis is to further evaluate the genetic variation of 34 provenances of trembling aspen and three hybrids, and the results were compared with those by cambium visual scoring (CVS). Provenance effects were significant, and the correlations between chlorophyll fluorescence and cambium visual scoring were significant.

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INTRODUCTION

Aspen (*Populus tremuloides* Michx), a deciduous hardwood species, is the most widely distributed native tree species in North American. It grows in a great diversity of regions and environments. Owing to its broad range of distribution, it exhibits marked genetic variation among and within populations throughout its range (Vaartaja, 1960; Barnes, 1969; 1975; Greene, 1971; Thomas *et al.* 1997).

Aspen has become economically more appealing to forest industries in North American in recent years. With its soft texture and short fiber, aspen has been used for lumber, pulp, paper products as well as veneer and plywood, fiberboard and particleboard and for many other uses. Breeding programs for aspen, mainly focusing on its adaptive variation, have started in the last few years in North America.

Cold hardiness, as one of the most important adaptive characteristics, is very critical to the survival and productivity of plants. Much research has been done on cold hardiness of important conifer and broadleaf tree species. However, no report on genetic variation in cold hardiness of aspen has been published.

A fast, simple and reliable method to test genetic differentiation in cold hardiness is very important for tree improvement programs. Electrical conductivity and visual scoring techniques are most often used, but these are either time consuming or subjective, respectively. Chlorophyll fluorescence has been demonstrated to be a simple, fast and reliable indicator of cold hardiness for conifer species. However, research on its

relative efficacy for broadleaf tree species is limited to bilberry and willow (ögren, 1996, 1999).

This study was based on seedlings of 32 provenances of *Populus tremuloides,* two of *P. tremula* L., and three *P.tremula* x *P. tremuloides* hybrids (Table 1). The purposes of this thesis were first to evaluate the relative efficiencies of chlorophyll fluorescence, electrical conductivity and cambium visual scoring techniques to detect genetic variation in cold hardiness among aspen provenances, and then to evaluate the adaptive variation in cold hardiness among aspen provenances by appropriate methods. To facilitate publication, this thesis is organized into three parts: (1) evaluation of chlorophyll fluorescence (CF), electrical conductivity (EC) and cambium visual scoring (CVS) for assessing genetic adaptive variation in cold hardiness among *Populus tremuloides* provenances from northwestern Ontario; (2) genetic adaptive variation in cold hardiness among *Populus tremuloides* provenances from northwestern Ontario assessed by electrical conductivity; and (3) Genetic variation in cold hardiness of *Populus tremuloides* , *P. tremula* and hybrid stem tissue assessed by chlorophyll fluorescence.

LITERATURE REVIEW

Low temperature represents, together with drought and salt stress, one of the most important environmental factors limiting the productivity and the distribution of plants on earth. Freezing is a widespread stress factor. On the global scale, only 25% of the total continental area of the earth can be considered frost free. For 64% of the earth's land, the minimum air temperature is below 0°C, for 48% below -10°C, and for 25% it is below -40°C (Sakai and Larcher, 1987). So research on genetic variation of cold hardiness and the selection of cold-hardy tree species or varieties possess important ecological and economic value.

FREEZING INJURY

Cold injury is one of the important factors limiting the production and the distribution of tree species. Reviews on this subject have been done by Levitt (1966, 1980), Mazur (1969) and Glerum (1976).

Freezing occurs in both hardy and non-hardy tissues. However, only the former can survive. According to Levitt (1980), there are two types of freezing occurring in plants: (1) extracellular freezing where the ice crystals form outside the cells and in the intercellular spaces; and (2) intracellular freezing where ice crystals form inside cells. Extracellular freezing predominates in nature and is non-fatal in hardy tissues, while the second type generally disrupts the cytoplasm and is nearly always fatal. The

second type occurs infrequently in nature (Glerum, 1976; 1985; Levitt, 1980).

Although many hypotheses have been advanced about freezing injury, the nature of freezing injury is not fully understood. Levitt (1962) demonstrated that the number of disulfide bonds increases with freezing injury and proposed a sulfhydryl (SH) hypothesis of freezing injury. According to this hypothesis, low temperature causes structural proteins to become reversibly denatured by unmasking reactive SH groups. However, there is no evidence that the increase of disulfide bonds associated with freezing injury completely results from low temperature. Mazur (1969) proposed a two-factor hypothesis of injury: at relatively fast cooling rates, injury is caused by intracellular ice formation; whereas at slow cooling rate, injury is caused by prolonged exposure to Solution Effects resulting from concentration of the intracellular solution or cell dehydration. Although this concept has received widespread acceptance, the inferred causality may be misleading (Steponkus, 1984). Ziegler and Kandler (1980) think that the plasma membrane plays a central role in cellular behavior during a freeze-thaw cycle, and the disturbance of the semi-permeable characteristics or lysis of plasma membrane and /or the tonoplast is a primary cause of freezing injury. The most accepted opinion now is that there are two main types of freezing injury: (1) primary injury due to intercellular freezing, and (2) secondary freeze-dehydration due to extracellular freezing. For extracellular ice formation, the fundamental cause of freezing injury is cell dehydration which leads to membrane disruption. For intercellular ice formation, the main cause is physical contact of the membrane with ice (Steponkus, 1984; Glerum, 1985).

NATURE OF COLD HARDINESS

The cold hardiness of a tree can be defined in general terms as the lowest temperature below the freezing point to which a tree can be subjected without being damaged (Glerum, 1976). The phenomenon, which enables a tree to increase or decrease its cold hardiness, is called the cold hardening process (Glerum, 1976). Frost hardiness is vital for the survival of plants growing in climates where freezing occurs. A lot of research has been done on nature of the cold hardiness process; however, little progress has been made toward elucidating its mechanism. Some results are even contradictory. These results reflect the complexity of cold hardiness, which is likely related to numerous biochemical and physiological processes (Glerum, 1976; Levitt, 1980).

In terms of cold resistance, there are essentially two main adaptive strategies available to the plants: avoidance and tolerance. Levitt (1978) lists six avoidance strategies and states that most of them must be accompanied by freezing tolerance of some tissues, in order for a plant to survive. Since tolerance of intracellular freezing is non-existent in nature as far as is known, the only tolerance to freezing strategy that can be developed by plants is extracellular freezing tolerance.

Several observers believe that frost hardiness occurs in two to three stages in woody plants native to temperate zones. According to Weiser (1970) and Sakai and Larcher (1987), the first stage of hardening is induced by short days, and the most important synthetic processes during this stage are accumulation of reserve starch and neutral lipids. The second stage is induced by temperatures just below 0°C, and it is in this stage that cold hardiness is greatly increased. The third stage is induced by

temperature of -30°C to -50°C. Only extremely hardy species are able to attain this stage, and this kind of hardiness is quickly lost at higher temperatures. Glerum (1985) demonstrates that the first stage occurs in early fall and is associated with the cessation of growth and development.

There is some disagreement about whether dormancy is a prerequisite for the development of cold hardiness. Weiser (1970) thinks that growth cessation rather than dormancy is the major factor in initiation of cold hardiness. Glerum (1976), however, emphasizes that both development and loss of cold hardiness lag behind the induction and loss of dormancy, and concludes that dormancy is a prerequisite for cold hardiness, at least in some species.

Cold hardiness changes noticeably in response to several environmental factors, such as temperature, availability of light, humidity, and even of CO_2 concentration (Sakai and Larcher, 1987). This is why plants of the same species may exhibit different degrees of hardening at different localities or at the same site in different years. Frequently, tissue water content is inversely related to hardiness. A slight difference in moisture content of hardy tissues has been demonstrated to have a significant influence on cold hardiness development. The water content of a plant influences its cold hardiness via the cell sap concentration and the degree of hydration of the protoplasm. Partial dehydration of woody stems can increase their hardiness by as much as 7°C (Burke *et al.* 1976). However, prolonged drought, which results in starvation, may weaken the plant and hence lead to a decrease in its cold resistance (Sakai and Larcher, 1987). Early investigators assumed that the total amount of bound water in close association with macromolecules was an important aspect of cold hardiness. Later

research by Levitt (1969) and Brown *et al.* (1970) has cast doubts on the importance of bound water to the hardening process. According to McGuire and Flint (1962), light increases the photosynthetic reserves of conifer seedlings during hardening, and they suggest that light is indispensable in the hardening process.

Many good positive correlations have been found between cold hardiness and the concentration of chemical substances such as sugars, proteins, lipids, and nucleic acids in tissues (Siminovitch *et al.*, 1968; Pomeroy *et al.*, 1970). However, as pointed out by Glerum (1976), these correlations rarely apply to these substances simultaneously and frequently they are correlate poorly during the dehardening period. In recent years, increasing attention has been focused on the cell membrane and its chemical composition. In general there is an increase of phospholipids and in the degree of unsaturation of the fatty acids during cold hardening process. Comparative study reveals characteristic difference in the membrane lipid composition between somewhat hardy, hardy and very hardy needles (Senser and Beck, 1984). These researchers found that the membrane lipid content of spruce needle after hardening in winter was almost twice that of their freezing-sensitive state in summer.

METHODS OF EVALUATING COLD HARDINESS

Cold hardiness is a general term for the resistance of a plant to freezing. Several methods have been used to evaluate cold hardiness. In general, these methods consist of two parts: a freezing treatment and the evaluation of the resulting injuries.

Freezing Test

Freezing tests can be conducted either in a field trial or in a laboratory. Despite their advantage of relating the results of such investigations to actual field injury, field trials have been proven to be slow and inaccurate owing to complex conditions. Frost damage in field trials can not be separated from the effects of other environmental factors.

A laboratory test is designed to simulate natural frost events while reducing the interference from other environmental factors. This kind of test is mainly conducted in a freezing chamber, but liquid bath and temperature gradient bars are also used (Timmis, 1976; Keates, 1990). The use of a freezing chamber was introduced by Harvey in 1918 (Levitt, 1980). Since then, many improvements have been made. These improvements include: aluminum shelf (Gusta *et al.*, 1978) and wide-mouthed large vacuum bottles (Harrison *et al.*, 1978) used to control temperature variation inside the freezer, and electrical controllers used to control the rate of freezing and thawing.

Materials for frost hardiness tests generally come from two sources: (1) field-grown trees and (2) seedlings grown in a nursery/greenhouse. The testing material can be shoot segments of current year growth (Colombo *et al*, 1984), detached leaves/needles (Hallam and Tibbits, 1998), buds (Simpson, 1994), roots or even the whole seedling (Nillsson and Anderson, 1987; Keates, 1990; Sundblad, 1990). Tests focused on plant tissues or organs have provided detailed information about hardiness differences between tissue types, and such tests are suited to large sample size (Keates, 1990). However, the low temperature tolerance of different tissues varies over the

growing season. Only measuring the hardiness of one kind of tissue may not be a reliable means for assessing the whole plant hardiness (Keates, 1990; Simpson, 1994). For this reason, a whole plant test is a more desirable method to evaluate cold hardiness than evaluation based only on one tissue.

Considerable research has been done concerning the freezing treatment of plant materials. Such research has mainly focused on the following topics: (1) the storage of materials prior to testing. In order to balance the sample temperatures, generally tissue samples should be maintained in a dark, 0°C to 5°C environment for 3-5 hours (Sakai and Weiser, 1973; Colombo *et al*, 1984); (2) point or step testing. Most experiments used point testing, where samples were treated at a single selected minimum temperature (Rehfeldt, 1979; Green and Warrington, 1978; Colombo *et al.*,1984). An alternative method, step testing, is designed to simulate a stress response over a temperature continuum (Hawkins *et al.*, 1994). By this method, a test group is divided into temperature subsamples, and each subsample is removed from the freezer following a timed exposure to progressively decreasing temperatures. One advantage of this method is that it can reduce variation between freezers; and (3) temperature regime. According to Keates (1990), prior to cold hardiness testing, it is critical to expose samples to a standard temperature (generally between 3°C to 10°C) to equilibrate the temperature of samples. Following that, a standard cooling rate of 5°C to 6°C per hour is recommended; the duration of test temperature exposure should be 1 to 3h; and, the rate of rewarming can be much faster than the rate of freezing. A range of 5°C to 20°C per hour is recommended for rewarming rate.

Evaluating Injury

After low temperature treatment, the next step is to evaluate freezing injury from cold temperature exposure. The methods of assessing the cold hardiness of tree seedlings can be divided into five major categories (Timmis, 1976): morphological methods, physiological methods, chemical methods, physical methods and electrical methods. Among them, morphological and electrical methods are most widely applied.

Morphological methods include visual inspection of needle/leaf, cambium, bud or root viability, after the tissue samples or whole seedling have been held for a standard duration (usually one week) in a controlled post-freeze environment. Ritchie (1984) states that the only way to unequivocally evaluate frost injury is to hold the seedlings in a greenhouse or growth chamber for a standard time, and then to inspect them visually for damage. However, this approach has several disadvantages: (1) assessments are subjective and difficult to standardize; (2) results are not directly quantifiable, and therefore data analysis is problematic; (3) the results do not necessarily relate to field survival under similar conditions. For example, bud browning may not result in seedling death unless a large proportion of buds are killed; and (4) visual assessments are time-consuming.

Various electrical assessment methods have been developed for evaluating cold hardiness in plants. Wilner (1960) provided a concise and comprehensive description of electrical methods. Among them, electrical conductivity (EC) and electrical impedance (EI) are widely used in practice.

Measurements of EI of plant tissues have long been used in physiological

studies, mainly for determining the extent of freezing injury (van Den Driessche, 1973; Timmis, 1976; Glerum, 1980; Shcherbinin and Lobanov, 1987; Keates, 1990). It is a rapid and non-destructive method for determining freezing injury in tissues of woody plants (Glerum, 1980). However, this method is influenced by a number of physical factors, such as tissue size, temperature, and moisture, which limit the application of this method in the forest.

The EC method is gaining popularity in operational cold hardiness testing of both coniferous and broadleaf tree species. EC measurements have been found to accurately indicate frost injury in Monterey pine (Ritchie, 1984), black spruce (Colombo *et al.*, 1984), jack pine (Colombo and Hickie, 1987), white spruce (Colombo *et al.*, 1982; Simpson, 1994), yellow-cedar (Hawkins *et al.*, 1994), and eucalyptus (Hallam and Tibbits, 1988), and some other tree species. Freezing disrupts cell membranes, causing leakage of the cell's electrolytic contents. The resulting freezing damage can be determined by immersing the shoots or needles in water for a defined length of time and the increase in the solution conductivity will be proportional to the amount of the damage (Colombo *et al.*, 1982). Colombo *et al.* (1984) described a standard procedure for measuring cold hardiness requiring three days to complete. Colombo and Hickie (1987) have shortened the time to one day through modifications of the method. The conductivity measurements are usually expressed as relative conductivity (RC) or index of injury (I_t). A high RC or I_t represents high freezing damage.

The EC method offers several advantages over physiological, chemical, or physical methods of frost hardiness assessments. EC methods are fairly rapid (usually three days), sensitive, and easy to conduct in operational use. However, the

interpretation of index of injury is problematic and depends on species, tissue, age, and even physiological state.

Chemical methods are used to assess chemical changes as a result of the breakdown of the photosynthetic apparatus or the inactivation of photosynthetic enzymes. They are also used to evaluate the effects of frost injury (Timmis, 1976; Keates, 1990). The main chemical methods include the tetrazolium chloride test (TTC), chlorophyll fluorescence (CF) and ethane production. Timmis (1976) found that TTC method could only accurately predict cold hardiness of somewhat hardy tissues of Douglas fir. Johnson and Gagnon (1988) evaluated ethane production as a method for assessing low temperature effects on *Pinus taeda* L. seedlings. They found that ethane production was linearly related to freezing temperature and electrical conductivity. However, MacRae *et al.* (1986) did not find a linear relationship between ethane production and freezing temperatures in non-coniferous species. These results show that further investigation of the ethane production method is needed to determine its efficacy.

In the last ten years, chlorophyll fluorescence (CF) has become a promising method to assess cold hardiness of tree seedlings. It is a simple, fast and nondestructive indicator of seedling dormancy and freezing tolerance. A detailed discussion of variable chlorophyll fluorescence theory and kinetics is provided by Krause and Weis (1991). In general, chlorophyll fluorescence may be defined as the red light (685-740μm) produced by photosystem II of the chloroplast thylakoid membrane when a plant is exposed to light (Bidwell, 1979; Binder and Fielder, 1996). The chlorophyll fluorescence variables, especially the ratio of variable fluorescence to maximal fluorescence (Fv/Fm) which

describes the photochemical efficiency of photosystem II, have been successfully used to assess cold hardiness of poplar (*Populus* spp.) (Havaux *et al.*, 1988), white spruce (Binder and Fielder, 1996), Douglas fir (Hawkins and Lister, 1985; Fisker et al., 1995), black spruce (Devisscher *et al.*, 1995), Scots pine (Sundblad, 1990; Lindgren and Hallgren, 1993); lodgepole pine (Lindgren and Hallgren, 1993), bilberry (Ögren, 1996) and willow (Ögren , 1999). Generally, good correlations have been indicated between cold hardiness estimated by CF and visual scoring or EC in those studies. However, any external or internal factor that can potentially affect the photosynthetic rate will cause corresponding changes in fluorescence emission (Vidaver, 1991; Binder and Fielder, 1996). Haranghy (1990) made a detailed investigation on the effects of external factors on the induced chlorophyll fluorescence response, and found that dark adaption time, previous light history, measuring time of day, excitation light intensity, exposure to light between dark adaption and testing and temperature could significantly affect the induced chlorophyll fluorescence responses of spruce seedlings. Devisscher *et al.* (1995) measured the Fv/Fm ratio of black spruce at three different times of the day (early morning, midmorning and afternoon) and found that stable readings could only be obtained between 8 to 9am.

Physiological methods mainly include infrared gas analysis (IRGA) and leaf segment floatation. Physical methods include nuclear magnetic resonance (NMR) and differential thermal analysis (DTA). On a practical level, however, further modifications are necessary for all those methods. More detailed description of these methods can be found in Timmis (1976) and Keates (1991).

Cold hardiness is a genetically controlled physiological trait. The different responses to low temperature form the basis for the differences between individuals, ecotypes, varieties and species. The gene pools of populations exhibit a range of variation both in hardening potential and seasonal timing, enabling populations to survive a certain degree of change in the environment (Sakai and Larcher, 1987).

Significant differences in cold hardiness between tree species have been demonstrated. From extensive cold hardiness studies on conifers growing in different parts of the world, large differences in winter hardiness are evident among families and genera (Sakai, 1983). Kozlowski (1979) also found great variation in cold hardiness among forest tree species: northern species, including *Populus tremuloides* and *Larix laricina* K.Koch, can resist temperature in midwinter to -80°C or lower. In contrast, southern species, such as *Quercus virginiana* P. Mill and *Magnolia grandiflora* L. develop cold hardiness only to -15°C. The timing of cold hardiness differs significantly among species, occurring earlier in those from northern or cold regions than those from south or warmer regions. Similarly, variations in cold hardiness of individuals and populations of a single species were different.

Similarly, variations in cold hardiness of individuals and populations of a single species have been detected. The provenance test is a good method to assess geographic and ecotypic variation in cold hardiness. Information on genetic adaptive variation among populations is very important to seed transfer. Rehfeldt (1979) found that variation in cold hardiness of *Pseudotsuga menziesii* (Mirb) var. *glauca* (Beissn) Franco

populations in the first stage of cold hardiness was related to geographic and ecological factors of the seed sources. Cold hardiness increased in *Pseudotsuga menziesii* Franco var. *glauca* Franco populations from higher latitude and higher altitude. However, in the second stage of cold hardiness, no such cline existed between seed sources and cold hardiness. Cannell and Sheppard (1982) studied seasonal changes in cold hardiness of *Picea sitchensis* (Bong.) Carr. in Scotland and found that the greatest difference between provenances occurred in the autumn.

Some common genetic adaptive variation trends in cold hardiness can be summarized as follows: (1) significant differences in cold hardiness exist among provenances, families or clones (Johnson *et al.*, 1986; Hawkins *et al.*, 1994). These differences form the basis for selecting frost-resistant materials; (2) the variation in cold hardiness among populations is strongly related to geographic variables of the place of origin. In general, northern populations are more resistant than the southern ones in autumn and winter. Better development of cold hardiness of northern populations is often associated with early cessation of growth (Lacaze, 1964; Flint, 1972; Campbell and Sorensen, 1973; Alaxander *et al.*, 1984; Joyce, 1987; Simpson, 1994; Hanners *et al.*, 1999). According to Eiga and Sakai (1982), freezing resistance of *Saghalien* fir increases with increasing altitude from about 300m to 1200m, and the increase in freezing resistance is always accompanied by a decrease in the variance. The drop in temperature associated with rising altitude brings with it an increase in frequency and degree of frost to which the trees are exposed. This increase evidently reflects the results of natural selection (Lacaze, 1964; Searle, 1991). However, exceptions do exist in some tree species (Haverbeke, 1979; Hawkins *et al.*, 1994); and, (3) the genetic differentiation

in cold hardiness among populations was under the control of climatic variables (Rehfeldt, 1979; Alexander *et al.*, 1984; Parker and van Niejenhuis, 1996; Balduman *et al.*, 1999). The varied significant associations between frost injury and gradients of climate in these reports indicate that variation among populations may be partially attributed to the combined effect of climatic variables.

GENETIC RESEARCH ON ASPEN

Owing to its broad range of distribution, aspen exhibits marked genetic variation throughout its range. Barnes (1969; 1975) studied phenotypic variation of leaves of aspen from south Utah and Colorado northward to the Canadian border, and found that there was a great deal of variation within areas, but the difference between areas was even more striking. Through research on local variation among clones, Greene (1971) concluded that aspen is morphologically, and presumably genetically, most uniform at its lowest and highest elevations. The greatest variation in leaf form occurs at intermediate elevations. Vaartaja (1960) compared growth responses under different photoperiods between southern and northern provenances, and concluded that the greater weight of the root system of northern aspen provenance is a genetic adaptation to cold soils. By studying natural stands of aspen in Wisconsin and northern Michigan, Einspahr and Benson (1967) found that there was a well-defined south to north trend of decreasing specific gravity of the sapwood, but no significant geographic trend of any growth traits was found. Thomas *et al.* (1997) concluded that trembling aspen in Alberta exhibited substantial genetic variation at the clone level for gas exchange traits, but with

limited differentiation at the population level. Yeh *et al.* (1995) described the population structure of trembling aspen with isoenzyme data, and found that the within-population component accounted for 97.4% of the total variation. By using isoenzyme techniques on 200 *Populus tremuloides* clones selected from eight populations across Ontario, Hyun *et al.* (1987) demonstrated that within population differentiation accounted for 79% of the genetic variability. They concluded that *Populus tremuloides* in Ontario is genetically variable and moderately differentiated.

Perhaps because aspen has become economically appealing to forest industries in North American only in recent years, research on genetic variation of aspen has not been systematic and complete. Most of our insight into genetic variation in aspen has come from studies of phenotypic variation among natural clones. Aspen provenance tests were only started in recent years (W. Parker, personal communication). Research on cold hardiness of aspen has not been reported until now.

EXPERIMENTAL MATERIALS

One-year old aspen seedlings grown from thirty-seven seed sources were planted in three provenance tests in northwestern Ontario in June, 1998. Among them, twenty six of the sources were collected from northwestern Ontario by Lakehead University. One source was provided by Weyerhaeuser at Alberta and ten additional sources were provided by the Aspen-Larch Genetics Cooperative, including three from Alberta and Saskatchewan, one from Minnesota, two European sources of *Populus tremula* L. and three full sib hybrid families of P. *tremula* x P. *tremuloides* (Table 1). Locations of the twenty-six northwestern Ontario sources are shown in Figure 1.

Locations of the three test sites are also shown in Figure 1. The first test is referred to as Camp 45 (C45), a more northerly boreal forest site. The second test is Dog River (DR), a more southerly boreal mixed forest site. The third test site, Kreikmann (KRKM) is a former hay field and sod farm near Kakabeka Falls, Ontario.

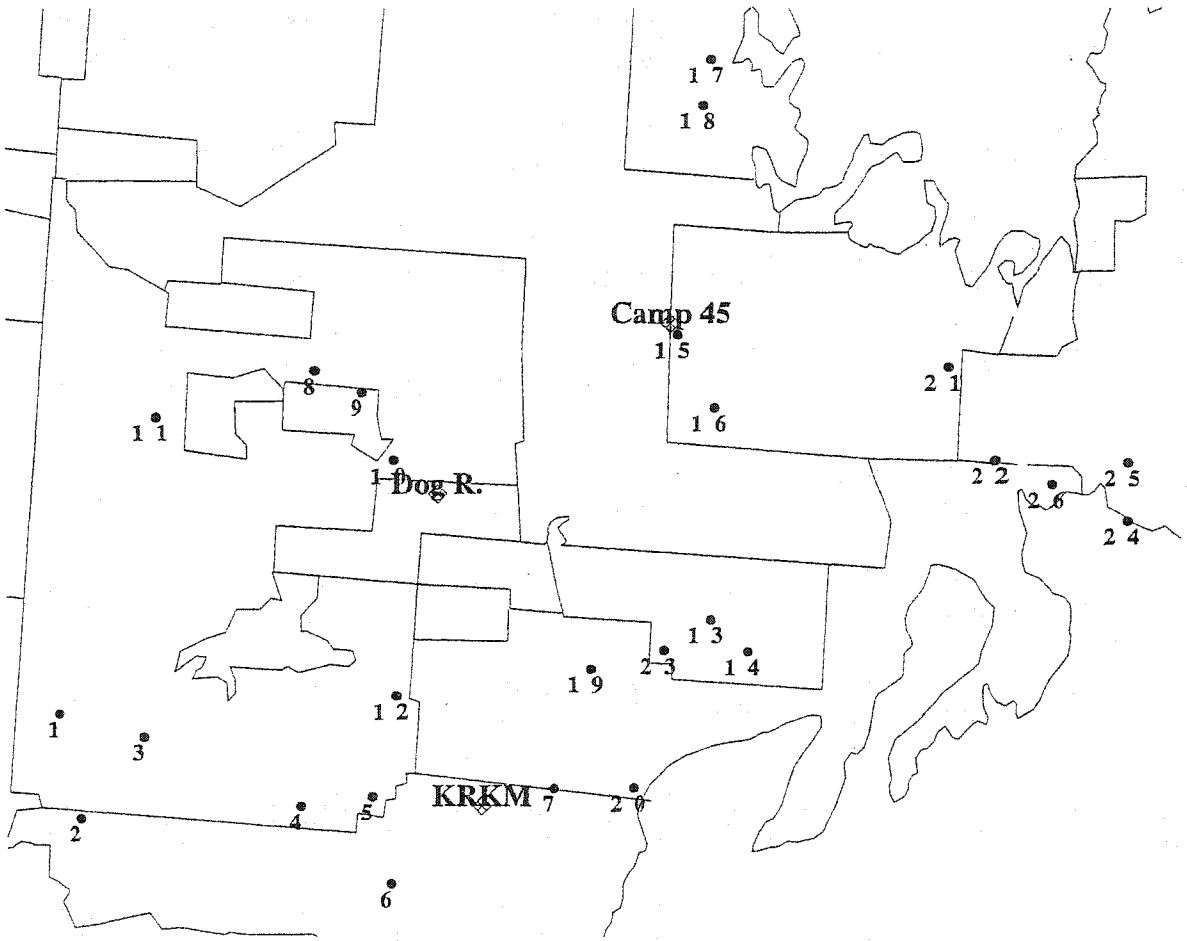
Seedlings were planted in a completely randomized experimental design, with three blocks at each of the three sites. Each block contains 15 replications (single tree plots) for each provenance. In addition, each block was surrounded by two rows of buffer trees to minimize edge effects. These buffer trees were 'extras' obtained from the twenty-six Ontario sources.

This thesis is organized into three parts, each with different focus. Part I

Table 1. Identification and geographic location of aspen seed source used in parts I, II and III

I	II	III	Source	Identification	Latitude	Longitude
•	•	•	1	Northwestern Ontario	48°29'35 N	90°48'57 W
•	•	•	2	Northwestern Ontario	48°18'20 N	90°44'05 W
•	•	•	3	Northwestern Ontario	48°27'45 N	90°35'02 W
	•	•	4	Northwestern Ontario	48°21'20 N	90°09'00 W
	•	•	5	Northwestern Ontario	48°22'55 N	89°57'35 W
		•	6	Northwestern Ontario	48°13'30 N	89°53'40 W
	•	•	7	Northwestern Ontario	48°25'03 N	89°28'23 W
•	•	•	8	Northwestern Ontario	49°09'02 N	90°11'29 W
•	•	•	9	Northwestern Ontario	49°07'00 N	90°03'34 W
•	•	•	10	Northwestern Ontario	48°59'49 N	89°57'44 W
		•	11	Northwestern Ontario	49°02'45 N	90°37'00 W
	•	•	12	Northwestern Ontario	48°34'05 N	89°54'50 W
		•	13	Northwestern Ontario	48°44'24 N	89°04'33 W
	•	•	14	Northwestern Ontario	48°41'07 N	89°58'13 W
	•	•	15	Northwestern Ontario	49°15'20 N	89°12'16 W
		•	16	Northwestern Ontario	49°07'34 N	89°05'47 W
•	•	•	17	Northwestern Ontario	49°45'42 N	89°09'26 W
•	•	•	18	Northwestern Ontario	49°40'34 N	89°10'17 W
•	•	•	19	Northwestern Ontario	48°38'19 N	89°23'29 W
	•	•	20	Northwestern Ontario	48°25'35 N	89°15'18 W
		•	21	Northwestern Ontario	49°13'19 N	88°27'37 W
		•	22	Northwestern Ontario	49°03'23 N	88°19'24 W
•	•	•	23	Northwestern Ontario	48°40'47 N	89°11'43 W
	•	•	24	Northwestern Ontario	48°57'13 N	87°58'13 W
•	•	•	25	Northwestern Ontario	49°03'38 N	87°58'28 W
•	•	•	26	Northwestern Ontario	49°00'57 N	88°10'38 W
		•	27	Aspen-larch Co-op MN Check –Itasca Co., Minnesota		
		•	28	Aspen-larch Co-op WI+MI-Oneida City, Wisconsin &Iron city, Minnesota		
		•	29	WeyerHaeuser_Drayton Valley, Alberta		
		•	30	Aspen-larch Co-op 68-92-*Populus tremula*, Central Poland		
		•	31	Aspen-larch Co-op 69-92-*Populus tremula*, Central Poland		
		•	32	Aspen-larch Co-op 69-93-Prince Albert, Saskatchewan		
		•	33	Aspen-larch Co-op 58-94-Peace River, Alberta		
		•	34	Aspen-larch Co-op 78-95-Slave Lake and Whitecourt, Alberta		
		•	35	Aspen-larch Co-op 20-96-*P.tremula* x *P.tremuloides*, Dickinson City, Michigan		
		•	36	Aspen-larch Co-op 21-96-*P.tremula* x *P.tremuloides*, Ontanagon City, Michigan		
		•	37	Aspen-larch Co-op 22-96-*P.tremula* x *P.tremuloides*, Alston, Michigan		

Note: provenance with '•' was included in study



● 1997 Seed Source Locations

◈ Test Site Locations

Figure 1. Aspen provenance locations from northwestern Ontario and test site locations

focused on the comparison of the relative efficiency of using chlorophyll fluorescence, electrical conductivity and cambium visual scoring to assess aspen cold hardiness. Twelve provenances from northwestern Ontario (Table 1 and Figure 1) were included. The purpose of Part II was to evaluate adaptive variation in cold hardiness among aspen populations from northwestern Ontario (Table 1 and Figure 1). Due to limited materials, only twenty provenances from northwestern Ontario were included. The objective of Part III was further to assess genetic variation in cold hardiness of aspen stem tissue among thirty seven provenances by chlorophyll fluorescence; thus, all provenances and hybrids were included in this study (Table 1).

PART I : COMPARING CHLOROPHYLL FLUORESCENCE, ELECTRICAL CONDUCTIVITY AND CAMBIUM VISUAL SCORING FOR ASSESSING ASPEN GENETIC VARIATION IN COLD HARDINESS

INTRODUCTION

Cold hardiness is an important adaptive trait in boreal forest tree species. Freezing damage can occur at almost any time of year in northwestern Ontario, but it is particularly common and widespread just after bud-burst in April-May and after growth cessation in September-October (Levitt, 1966; Glerum, 1985). An extensive literature exists on genetic variation in cold hardiness within tree species. Large provenance differences in cold hardiness have been reported in many species, including Douglas-fir (Larsen, 1978; Rehfeldt, 1979), Sitka spruce (Cannell and Sheppard, 1982) and white spruce (Simpson, 1994). Such differences among provenances can often be closely related with geographic or climatic variables at the sites of origin (Simpson, 1994; Parker and van Niejenenhuis, 1996).

Cold hardiness can be determined by controlled freezing tests. The resulting frost damage can be assessed by a number of methods, such as visual assessment of the damage, electrolyte leakage or electrical impedence. There are disadvantages to these methods in that they are destructive, subjective or require a relatively long time to quantify the damage. A simple, rapid, non-destructive and reliable method of ranking provenances, progeny or clones according to their cold hardiness would be of

considerable value in forest genetic programs.

In the past decade, chlorophyll fluorescence (CF), a measure of the status of the photosynthetic mechanism, has been widely used in studies to determine how environmental factors, e.g. freezing, alter the photosynthetic capacity of plants (Haranghy, 1990). CF can be used to monitor cellular injury caused by environmental stress rapidly and non-destructively and to determine the relative stress tolerance of different species (Smile and Hetherington, 1983). CF has been successfully used to assess cold hardiness in many evergreen conifer tree species (Binder and Fielder, 1996; Hawkins and Lister, 1985; Fisker *et al.*, 1995; Devisscher et al. 1995; Sundblad *et al.*, 1990 and Lingren and Hallgren, 1993). These works have demonstrated significant correlations between chlorophyll fluorescence and visual assessment or electrolyte leakage measure of freezing injury. However, for deciduous tree species like aspen, it is difficult to collect leaves in late September or early October when leaves are senescing; this is a critical period to test genetic differentiation in cold hardiness in northwestern Ontario. In studies of heterogeneous stems of bilberry and willow, Ögren (1996, 1999) found high correlations between cold hardiness assessed by chlorophyll fluorescence and electrical conductivity or visual scoring. He concluded that chlorophyll fluorescence method is a non-destructive, reliable and fast method to test cold hardiness of detached stems.

Provenance tests provide a means to assess geographic and ecotypic variation in cold hardiness. The intent of this study was to evaluate chlorophyll fluorescence as a suitable and quick measure of genetic variation in cold hardiness using materials from two aspen provenance tests established in northwestern Ontario. To accomplish this

goal, the results obtained from chlorophyll fluorescence were compared with those obtained by the electrical conductivity test (EC) and cambium damage visual scoring (CVS) -- two most often used methods in Ontario. Since cold hardiness is an adaptive trait in boreal species, patterns of frost hardiness variation among provenances should correlate with climate. A second goal of this study was to compare the relative efficacy of each of the three methods to test adaptive variation by the strength of the correlation to climatic data obtained for each provenance seed source location. Since frost hardiness trials were carried out for two successive seasons, a further goal was to establish the repeatability of the results obtained by chlorophyll fluorescence.

MATERIALS AND METHODS

Frost Hardiness Data

One-year old aspen seedlings grown from seeds collected at 12 locations (1, 2, 3, 8, 9, 10, 17, 18, 19, 23, 25 and 26) representing four regional groups of three sources from northwestern Ontario were planted in two provenance tests in northwestern Ontario in June, 1998 (Figure 1 and Table 1). The provenance tests are referred to as Camp 45 (C45), a more northerly boreal forest site, and Kriekmann (KRKM), a more southerly black spruce seed orchard location established on an old sod farm near Kakabeka Falls, Ontario. Twenty seedlings of each of the 12 provenances were randomly sampled three times in the fall of 1999, and twice more in the fall of 2000. Sampling dates were: i) September 5, 1999 at KRKM, ii) September 15,1999 at Camp

45, iii) October 5 and 6, 1999 at KRKM and Camp 45, iv) September 15, 2000 at Camp 45, and v) October 1, 2000 at Camp 45. At each sampling date three healthy lateral branch (each about 5 to 6 cm in length) of the current-year growth were taken from the middle part of the seedling and two healthy leaves from the upper stem were collected from each tree, sealed in wet plastic bags (containing wet papers), transported to the laboratory and kept at 5°C overnight. Freezing treatments were carried out the next day in a programmable freezer. For chlorophyll fluorescence and visual scoring, intact branch or leaves of each provenance were equally divided into 3 or 4 uncovered paper bags depending on the number of temperatures used at each sampling date. For electrical leakage, each branch was cut into three approximately 1 cm segments from the apical bud of twig, and the segments from each origin were equally divided among 9 uncovered test vials, with three vials for each treatment temperature. One bag and three vials of sample were kept at 5°C as controls, and the others were placed in a programmable freezer. The freezing temperatures varied from -10°C to -40°C depending on the date (Table 3). Several thermocouples were used to monitor the temperature inside the freezer but not inside the vials or bags. Preliminary tests indicated that the temperatures inside uncovered paper bags or test vials were the same as that of the air in the freezer. After half an hour at +5°C, the temperature was lowered at a rate of 6°C per hour to the preset test temperatures and held for one hour. Then the temperature was increased by 10°C per hour to +5°C. Following freezing, cold damage was determined by the three methods.

The Plant Stress Meter (FMS-2 Fluorescence Monitoring System, Hansatech, England) was used to measure the fluorescence ratio Fv/Fm of leaf and stem tissue. All

leaves and branches were dark-adapted for 2 hours (20°C) before measurement. Measurements were carried out under dark conditions. During measurement, the leaf (upper side) or lateral branch (upper side, the side exposure to sunshine) located just below the terminal bud was held directly against the fiber optic probe and exposed to a blue excitation beam of 3000 μmol/m^2/s. The fluorescence variable Fv/Fm ratio was recorded. The Fv/Fm ratio was selected as the response variable in this study since it has been demonstrated to be a good indictor of cold hardiness of plants (Lindgren and Hallgren, 1993; Fisker *et al.*, 1995). Leaf chlorophyll fluorescence was measured in 1999 and 2000, while stem chlorophyll fluorescence was measured only in 2000.

Stem frost hardiness was also determined using an electrical conductivity (EC) test (Colombo *et al.* 1984). Twenty ml of deionized water was added to each vial after freezing. After twenty-four hours the solution conductivity for both the control samples ($EC_{(control)}$) and frozen samples ($EC_{(frozen)}$) were measured. The stems were then autoclaved at 110°C for 1 hour and kept at room temperature for another 24 hours, after which the solution conductivity ($EC_{(killed)}$)was re-measured. Frost hardiness was expressed as relative conductivity (RC) (Colombo *et al.* 1984):

$$RC_{(frozen)} \text{ (or } RC_{(control))}) = EC_{(frozen)} \text{ (or } EC_{(control)}) / EC_{(killed)};$$

Higher values of RC indicate lower frost resistance. Stem frost hardiness estimated by electrical conductivity was determined only in 1999.

Cambium visual damage scoring (CVS) was also used to assess frost hardiness of stem tissue. After measuring the Fv/Fm ratio, stems were cultured in water at room temperature (20°C) for one week. Stem damage was assessed by slicing the stem lengthwise with a razor blade and measuring the length of discolored stem tissue (xylem

/cambium). The percentage of the length of discolored stem compared to total stem length was used as an indicator of cold hardiness. Visual scoring was conducted only in 2000.

Climate Data

All climate data for this study were derived from the Ontario Climate Model (OCM) (Mackey et al. 1996). This model is based on long-term monthly data (1951-1980) from 471 weather stations located in and around Ontario. Primary data of monthly mean, minimum and maximum temperatures and precipitation are provided by this model as well as a number of derived secondary climatic variables that are considered important in determining plant growth. Interpolation for the OCM was accomplished by coupling thin-plate smoothing spline surfaces fitted as a function of longitude, latitude and elevation to a one-kilometer resolution digital elevation model of Ontario (Mackey et al 1996).

For this study, Arc/Info software (Environmental Systems Research Institute 1987) was used to grid the OCM data for 65 climate variables at about 2 km resolution. Values for each of the climate variables were obtained for each of the 12 aspen seed source locations using the 'cellvalue' function in Arcplot. Table 2 presents a summary of these climate variables selected from those that proved to be important for predicting aspen frost damage. The five temperature variables vary from 0.8 to 1.6°C across the 12 seed source locations, while the precipitation variables show more variation ranging up to 14 percent more precipitation over the growing season for the moister sites. As well,

Table 2. Summary of climatic data obtained from the digital Ontario Climatic Model for 12 aspen seed source from northwestern Ontario

OCM Variable*	Minimum	Maximum	Mean	Coefficient of Variation
Maymnt (°C)	8.4	10.0	9.0	6.4%
Junmin (°C)	6.4	7.8	7.1	5.5%
Julmint (°C)	10.2	11.2	10.7	2.6%
Augmaxt (°C)	21.6	22.8	22.2	1.7%
Mnt3 (°C)	12.5	13.3	13.0	1.7%
GDD2 (>5°C)	222.0	257.0	243.5	4.3%
Growlng (days)	153.0	167.0	159.1	2.9%
Marprec (mm)	41.2	46.5	44.3	3.7%
Mayprec (mm)	63.9	75.1	69.4	5.6%
Augprec (mm)	85.0	91.2	88.1	2.1%
Prec1 (mm)	110.0	127.0	119.8	4.1%
Prec3 (mm)	419.0	490.0	447.9	5.6%

* Maymnt, mean temperature for May; Junmint, mean minimum June temperature; Julmint, mean minimum July temperature; Augmaxt, mean maximum August temperature; Mnt3, mean temperature for period 3--entire growing season; GDD2, growing degree days for period 2--the first six weeks of the growing season (heat sum>5°); Growlng, length of growing season; Marprec, mean March precipitation; Mayprec, mean May precipitation; Augprec, mean August precipitation; Prec1, mean total precipitation for period 1---before starting growing; Prec3, mean total precipitation for period 3---the entire growing season.

length of the growing season varied by as much as 14 days.

Data Analysis

SAS (1989) statistical software was used for all data analyses. Data were analyzed for provenance effects at each temperature of the freezing test at each provenance trial location by the linear model:

$$Y_{ij} = \mu + P_i + \varepsilon_{(i)j};$$

where Y_{ij} is the Fv/Fm ratio, RC or visual damage individual value, μ is the mean of Fv/Fm ratio, RC or visual damage, P_i is the random effect of the $_i$th provenance, and $\varepsilon_{(i)j}$ is the effect of the $_j$th replicate. Intraclass correlation coefficients (Sokal and Rohlf 1981) were calculated to determine the degree of differentiation expressed among the 12 seed sources. Comparisons between the results of the different assessment methods, and the relationship of the freezing results with climatic data for each of the seed origins were evaluated through correlation analysis and multiple linear regression. The 'maxr' option of SAS was used to determine significant ($p < 0.05$) regression based on the best one- to three-variable combination of climate factors; i.e., that combination having the highest coefficient of determination (r^2).

RESULTS

Chlorophyll Fluorescence--Leaf

Colder freezing temperatures decreased leaf Fv/Fm ratio (Table 3). The average leaf Fv/Fm ratio varied depending on site and freezing temperature. The absolute Fv/Fm values were slightly higher in plants from KRKM than C45 in 1999 except for the -20°C treatment, and also in 2000 compared to 1999 at all test temperatures at site C45. While the absolute values of the mean Fv/Fm values were somewhat different, the relative decrease in the values after freezing was remarkably consistent, first for the different freezing test dates within and between years, and second for the provenance test sites. The freezing treatments of -10°C resulted in a 13 to 17% decrease of Fv/Fm, while the -20°C treatment resulted in a decrease of 50 to 56 percent. Leaf Fv/Fm was not measured after middle September due to sampling difficulty, thus the seasonal development of cold hardiness expressed by leaf Fv/Fm could not be evaluated.

Variance components analysis on leaf freezing treatment indicated that the percentage of total variation expressed among provenances increased with colder temperatures (Table 3). Variation among provenances was highest at KRKM, reaching 41% after the -20°C treatment. Although the mean Fv/Fm values were comparable for Camp 45 samples in 1999 and 2000, the percentages of variance dropped 10% and 15% respectively in 2000 for the -10°C and -20°C treatments presumably due to an increase in experimental error or differences in weather between years.

Despite high genetic differentiation estimated by leaf Fv/Fm ratio, no significant predicting model was found in 1999 between this parameter and climatic variables characterizing the seed sources. However, in 2000, the leaf Fv/Fm ratios were highly correlated with climatic variables (r^2 is 0.77 and 0.72, respectively) (Table 4). The important climatic variables included mean maximum August temperature and mean

Table 3: Results of controlled freezing trials of aspen provenances based on three assessment methods.

Method	Tissue	Site	Date	T (°C)	Mean*(SE)	Reduction(%)**	r_i (%)***
EC	Stem	C45	Sept.15, '99	5	0.244 (0.003)		-
				-10	0.472 (0.007)	30	85.95
				-20	0.594 (0.008)	46	91.03
EC	Stem	C45	Oct. 6, '99	5	0.218 (0.004)		-
				-20	0.304 (0.006)	11	59.13
				-40	0.360 (0.005)	18	70.54
EC	Stem	KRKM	Sept.5, '99	5	0.306 (0.043)		-
				-10	0.502 (0.008)	28	87.15
				-20	0.645 (0.008)	49	59.32
EC	Stem	KRKM	Oct. 5, '99	5	0.243 (0.005)		-
				-20	0.265 (0.004)	3	53.79
				-40	0.337 (0.004)	13	75.77
CF	Leaf	C45	Sept.15,'99	5	0.817 (0.003)		-
				-10	0.683 (0.013)	17	23.31
				-20	0.395 (0.009)	52	35.01
CF	Leaf	C45	Sept.15,'00	5	0.757 (0.004)		-
				-10	0.659 (0.005)	13	13.45
				-20	0.378 (0.009)	50	20.13
CF	Leaf	KRKM	Sept.5,'99	5	0.824 (0.003)		-
				-10	0.715 (0.025)	13	28.29
				-20	0.364 (0.025)	56	41.25
CF	Stem	C45	Sept.15,'00	5	0.568 (0.005)		-
				-10	0.453 (0.006)	20	7.2
				-15	0.346 (0.007)	39	28.38
				-20	0.249 (0.008)	56	26.02
CF	Stem	C45	Oct. 1, '00	5	0.52 (0.005)		-
				-10	0.469 (0.009)	10	0
				-22	0.405 (0.004)	22	14.14
				-33	0.339 (0.005)	35	18.76
VS	Stem	C45	Sept.15,'00	-10	34.5 (21.20)	35	8.28
				-15	60.06 (7.91)	60	22
				-20	68.13 (14.51)	68	24.16
VS	Stem	C45	Oct.1, '00	-10	0.60 (0.344)	0	0
				-22	12.47 (1.146)	12	16.03
				-33	27.73 (2.015)	28	21.94

*mean of EC is RC; mean of CF is Fv/Fm ratio; mean of VS is percent damage.

**For EC, reduction(%) $=(RC_{(treated)} - RC_{(5°C)})/(1-RC_{(5°C)})*100$;
for CF, reduction(%) $=(CF_{(5°C)} - CF_{(treated)})/CF_{(5°C)}*100$.

***intraclass correlation coefficient--- the variation among provenances

Table 4. Multiple regression results between cold hardiness and climate variables in 1999

Site	Date	Method	Tissue	T	Independent Variable*	r^2	Prob.
C45	Sept.15, '99	CF	Leaf	-10°C		ns	
			Leaf	-20°C		ns	
		EC	Stem	-10°C	MarPrec, Prec3	0.616	0.013
			Stem	-20°C	MarMnT, MarPrec, Augprec	0.683	0.021
	Oct.6, '99	EC	Stem	-20°C	MnT3	0.629	0.002
			Stem	-40°C	AprMaxT, Prec3	0.641	0.010
KRKM	Sept.5, '99	CF	Leaf	-10°C		ns	
			Leaf	-20°C		ns	
		EC	Stem	-10°C	JulMinT, JulMnT, MayPrec	0.662	0.028
			Stem	-20°C	MayMaxT, JulMinT, MayPrec	0.650	0.031
	Oct.5, '99	EC	Stem	-20°C	SepMnT, OctPrec, GDD2	0.739	0.010
			Stem	-40°C		ns	

* Julmint, mean minimum July temperature; Aprmaxt, maximum April temperature; Maymaxt, maximum May temperature; Marmnt, mean temperature in March; Julmnt, mean temperature in July; Sepmnt, mean temperature in September; Mnt3, mean temperature for period 3--entire growing season; GDD2, growing degree days for period 2--the first six weeks of the growing season (heat sum>5°); Marprec, mean March precipitation; Mayprec, mean May precipitation; Augprec, mean August precipitation; Octprec, mean October precipitation; Prec3, mean total precipitation for period 3---the entire growing season.

Table 5. Multiple regression results between cold hardiness and climate variables in 2000

Site	Date	Method	Tissue	T	Independent Variable*	r^2	Prob.
C45	Sept.15, '00	CF	Leaf	-10°C	AugMaxT, JulMnT, MnT3	0.765	0.007
			Leaf	-20°C	AugMaxT, SepPrec, MnT3	0.720	0.013
			Stem	-10°C	MayMnT, GDD2 0.741	0.010	
			Stem	-15°C	JulMnT	0.765	0.000
			Stem	-20°C	OctMnT, AugPrec, GrowLng	0.768	0.007
		VS	Stem	-10°C		ns	
			Stem	-15°C	MayMinT, MayMnT, GrowLng	0.749	0.009
			Stem	-20°C	NovMaxT, JulMinT, NovMnt	0.830	0.002
	Oct.1, '00	CF	Stem	-10°C		ns	
			Stem	-22°C		ns	
			Stem	-33°C		ns	
		VS	Stem	-10°C	MarPrec, AugPrec, GrowLng	0.746	0.009
			Stem	-22°C		ns	
			Stem	-33°C		ns	

* Maymnt, mean temperature for May; Julmnt, mean temperature in July; Octmnt, mean temperature for October; Novmnt, mean temperature for November; Mnt3, mean temperature for period 3--entire growing season; Maymint, mean minimum May temperature; Julmint, mean minimum July temperature; Augmaxt, mean maximum August temperature; Novmaxt, mean maximum November temperature; GDD2, growing degree days for period 2--the first six weeks of the growing season (heat sum>5°); Growlng, length of growing season; Marprec, mean March precipitation; Augprec, mean August precipitation; Sepprec, mean September precipitation.

temperature for period 3 --- the entire growing season.

Chlorophyll Fluorescence--Stem

The use of chlorophyll fluorescence to determine the extent of freezing damage in stem tissue produced a consistent decrease of Fv/Fm with lower temperatures in 2000. Freezing on September 15, 2000 resulted in 20, 39 and 56% decreases from control values for -10°C, -15°C and -20°C, respectively, while on October 1 freezing resulted in 10, 22 and 35 % reductions for the same temperatures (Table 3). In the interval of two weeks, the seedlings had become more frost hardy; less damage was observed for the -22°C treatment on October 1 than for the earlier -20°C treatment on September 15.

As for leaf tissue, ANOVA results obtained for stem Fv/Fm indicated that the percentage of variation expressed among provenances increased with colder treatment temperatures. On September 15, variation decreased from 7% at -10°C to 28% at the lower treatment temperatures. However, the observed provenance differences decreased substantially by October 1, ranging from 0.0 percent at -10°C to less than 19% at the coldest treatment temperature. The percent of variation was greater for stem tissue than for leaf tissue collected on the same date (September 15), suggesting that stem tissue might be a slightly better indicator than leaf tissue for comparative provenance trial results.

In consistent with the results of leaf Fv/Fm in 2000, September experiments showed a strong correlation between stem Fv/Fm and climatic variables while in

October no clear agreement was found between them (Table 4). The correlation of September stem Fv/Fm against monthly mean temperature and precipitation had r^2 values of 0.741 to0.768 (Table 4). Thus, chlorophyll fluorescence could be used to detect genetic adaptive variation in cold hardiness of both leaf and stem tissue in September but not in October.

Visual Scoring-Stem

Decreasing freezing temperatures increased the visual freeze damage of stem (Table 3). As for the other two methods, the freezing damage was greatly reduced from September 15 to October 1, indicating that cold hardiness increased during the two week interval. The percentages of variation expressed among provenances that varied from 8.28% to 24.16% in September and from 0.00 to 21.94% in October were comparable with those estimated by chlorophyll fluorescence for the same test dates and freezing temperatures. As demonstrated by other methods in this study, the percentage of variance due to provenance was higher in September than October, suggesting that September is a better time to test genetic differentiation of aspen cold hardiness in northwest Ontario.

The regressions between visual damage and climatic variables were similar to the results for the chlorophyll fluorescence. In September, climatic variables unique to seed origin accounted for 75% and 83% of the variation in cold hardiness at -15°C and -20°C, respectively (Table 4). Temperature in May and November, together with the length of growing season, might be the most important factors controlling the genetic

differentiation in cold hardiness development in September. However, in spite of significant variation expressed among provenances, a significant predictive regression equation for cold hardiness was found for the October only at -10°C (Table 4).

Electrical Conductivity--Stem

Lower freezing temperatures increased damage, as estimated by EC. Although sampling date at Camp 45 was 10 days later than at KRKM in September, frost tolerance expressed by percent reduction of RC relative to control samples was quite consistent for the two sites with 30% at -10°C and 46% at -20°C. The percent reduction of RC relative to control samples decreased to 11% at -20°C and 18% at -40°C in October at C45, and 3% and 13% for the same temperatures at KRKM (Table 3). These results indicated that, in the interval of three-four weeks, the hardiness of seedlings increased greatly, and provenances at KRKM possessed higher cold resistance at that time. As with the other methods, percentages of variance in RC due to provenance were higher in September than in October, ranging from 60% to 91%. However, variation expressed among provenances was higher compared with other methods (Table 3). The percent variations expressed among provenances by RC value were about three times those detected by leaf Fv/Fm under same test conditions. These results were probably caused by relatively lower experimental errors for electrical leakage and suggested that this technique might be a better method to measure the level of genetic variation in cold hardiness.

Both September and October of 1999 treatments demonstrated strong correlation

between cold hardiness estimated by electrical leakage and the climatic variables of each seed origin except the -40°C treatment of KRKM samples, suggesting that this method could be used to test genetic adaptive variation both in September and October. However, the climatic variables retained in the predictive equations varied depending on site and freezing temperature (Table 4). Precipitation in March and mean precipitation for the entire growing season were the most important variables controlling genetic differentiation in cold hardiness among provenances at Camp 45, while temperature from May to July and precipitation in May were the most important factors predicting genetic variation in cold hardiness among provenances at KRKM. This difference may reflect the difference of gene expression of the cold hardiness development process under different environmental conditions.

Correlations Among Methods

Correlation analyses were conducted based on provenance means of Fv/Fm, RC and visual damage at each temperature and site (Table 5). High Fv/Fm, low RC or low visual damage values indicate high cold hardiness level. Thus, a negative association between Fv/Fm and RC or visual damage, or a positive relationship between RC and visual damage were expected.

Agreements between different freezing temperatures using the same method were positive and relatively strong as expected (Table 5), with most cases, the r value being greater than 0.6. However, correlations between methods varied depending on tissue type and sampling date. On the same sampling date, good correlations were found

Table 6. Pearson correlation coefficients (only >±0.3) among methods, sampling dates, tissues and sites

SITE			C45																				KM					
	DATE			15, Sept.,99				6, Oct.,99		15, Sept., 00							1, Oct., 00						5, Sept., 99				5, Oct., 99	
	Method			CF		EC		EC		CF				VS			CF			VS			CF		EC		EC	
			VAR	L-20	L-10	S-20	S-10	S-20	S-40	L-10	L-20	S-10	S-15	S-20	S-10	S-15	S-20	S-10	S-22	S-33	S-10	S-22	S-33	L-20	L-10	S-20	S-10	S-40	S-20
C45	5, Sept.	CF	L-20	1	0.57*	-0.5		0.32	0.48				-0.5		0.15	-0.4			0.74					0.51		0.36	0.09	0.09	0.32
	99		L-10		1				0.44		-0.4	0.55		-0.5	0.27			0.58	0.37	0.66	0.22					0.48	0.24	0.04	-0.3
		EC	S-20			1	0.69								-0.4				-0.6		-0.4	0.11	0.39	-0.4			-0.4	-0.4
			S-10				1								-0.6			0.38	-0.5									-0.7	-0.7
	6, Oct.	EC	S-20					1	0.74	-0.6	-0.5		-0.7	-0.7															
	99		S-40						1		-0.5		-0.8	-0.8			0.36					0.31			-0.4				
	15, Sept.	CF	L-10							1	0.77			0.47			0.35							0.33	0.26			-0.4	
	00		L-20								1		0.44	0.69	-0.4	-0.1	-0.3				-0.6						0.31	-0.3	
			S-10									1								0.52	0.33		0.37			0.41			-0.4
			S-15										1	0.66	-0.6	-0.5	-0.4								0.32	-0.3			-0.4
			S-20											1	-0.5	-0.4	-0.5							0.36	0.22				
		VS	S-10												1	0.53	0.73					0.36					0.36	0.77	0.56
			S-15													1	0.51									0.37	0.4		
			S-20														1						0.31					0.66	0.43
	1, Oct.	CF	S-10															1	0.39	0.73	-0.4			-0.3	-0.3				0.63
	00		S-22																1	0.5		-0.4	-0.6	0.43	-0.4	0.42	0.37		
			S-33																	1			-0.4		-0.5	0.53	0.41		0.6
		VS	S-10																		1	0.7	0.42				-0.5		
			S-22																			1	0.59			-0.5	-0.8	-0.4	-0.4
			S-33																				1			-0.3	-0.7		
KM	5, Sept.	CF	L-20																					1					0.56
	99		L-10																						1				
		EC	S-20																							1	0.7		
			S-10																								1		
	5, Oct.	EC	S-40																									1	0.71
	99		S-20																										1

between stem Fv/Fm and visual damage except at –10°C both in September and October of 2000, between leaf Fv/Fm and RC in September of 1999 at –20°C at site C45 (r = -0.51), and between leaf Fv/Fm and stem Fv/Fm in September of 2000 (r from 0.44 to 0.69). However, for most test combinations on the same sampling date, correlations between methods in 1999 and 2000 were poor or in some cases contradictory (Table 5).

Some strong correlations between 1999 and 2000 were demonstrated, as expected (Table 5). Significant negative correlations were found between RC in October, 1999 and stem Fv/Fm in September, 2000 (r range from -0.71 to -0.78) at same test site, and between RC at -20°C in September 1999 and stem Fv/Fm ratio at -22°C in October 2000 (r =-0.60).

DISCUSSION

A successful screening technique in a breeding program should be rapid, simple, and ideally, non-destructive of plant materials. Also, the technique should accurately detect the genetic adaptive variation. In this study, the relative efficacy of three methods used to test genetic variation in cold hardiness was compared on four levels: general trends, genetic information, genetic adaptive variation, and correlations among them.

General Trend

As expected, cold damage estimated by CF, EC or CVS increased as treatment temperature decreased. With decreasing treatment temperatures, leaf and stem Fv/Fm decreased, while RC and CVS increased, suggesting that tissue response to freezing was

consistently estimated by three methods. The consistency in the percentage reduction of leaf or stem Fv/Fm after freezing within and between years confirms results seen for other species (Wulf *et al.* 1994; ögren, 1996, 1999). Also, cold hardiness development process from September to October estimated by these three methods was similar. The significant reduction of RC, Fv/Fm and CVS from September to October suggests that the cold hardiness of aspen populations from northwest Ontario increased greatly in the 2-3 week interval. Thus, if the purpose is simply to understand the cold hardiness development or tissue response to cold damage, CF may be a preferred indicator of cold hardiness due to its simplicity, non-destructive nature and short time required for assessments. Generally, the CF procedure can be completed in one day, while EC and CVS need three days and seven days to finish, respectively.

Genetic Information

For most treatment combinations, significant provenance effects were demonstrated by EC, CF and CVS. Variation in cold hardiness expressed among provenances was higher in September than in October by all methods, suggesting that September is a better period to test genetic variation for fall cold hardiness of aspen in the study area. The percentage variance expressed among provenances varied depending on method used. Variation among provenances estimated by RC was higher than that estimated by CF and CVS. Percentages of variance expressed among provenances estimated by CF and CVS were comparable. Stem Fv/Fm were slightly higher than leaf Fv/Fm. By this criterion, EC is most sensitive to test genetic information in cold

hardiness of aspen populations in this area, followed by stem Fv/Fm.

Differences in variance expressed among provenances may be mainly caused by differencies in experimental errors between methods. The application of the standard procedure for measuring EC published by Colombo *et al.*(1984) may reduce experimental errors. Estimation of CVS is inherently subjective, which may greatly increase the experimental errors. CF can be affected by factors such as dark adaption time, previous light history, time of day, excitation light intensity, and exposure to light between dark adaption and testing, that can potentially affect the photosynthetic rate and cause corresponding changes in fluorescence emission (Vidaver *et al.*, 1991; Binder and Fielder, 1996; Haranghy, 1990). These results indicate that environmental control is important for accurate prediction of cold damage by CF, and changes in these factors could increase experimental error. Although, many experimental conditions and procedures have been reported for the chlorophyll fluorescence technique, so far no standard experimental conditions have been set up for applying the chlorophyll fluorescence technique to test cold hardiness.

Determination of the level of provenance differentiation in cold hardiness by the three methods may also reflect differences in repair mechanisms each method measures. EC measures the ability of cytoplasm and the cell membrane to withstand low temperature. Because EC measures primary damage, it might overestimate cold damage. CF measures the effects of freezing on photosynthetic activity in the chloroplast. Two hours' dark adaptation may partly repair some of damage as demonstrated by Strand and Öquist (1988) and Ögren (1996; 1999). CVS measures the effects of freezing on cambium tissue, the longer duration required for cambium visual scoring provides more

time and substrates for repair.

Comparison of Chlorophyll Fluorescence, Electrical Conductivity and Cambium Visual Scoring

An important finding of this study is the good correlations demonstrated between stem Fv/Fm and cambium visual damage after freezing below -10°C in 2000 (Table 5). These significant correlations support the inference that linear reductions in Fv/Fm reflect actual tissue injury and therefore CF could be a preferable method to rank provenance freezing damage since it is simple, objective, fast and non-destructive. Other good correlations were found between leaf Fv/Fm and stem Fv/Fm in year 2000, and between leaf Fv/Fm and RC in 1999, but only at specific temperatures.

Another important finding is the correlations between stem damage estimated by CF in 2000 and stem freezing damage estimated by EC in 1999. Significant negative correlations were found between RC in October in 1999 and stem Fv/Fm ratio in September in 2000 (significant r values ranging from -0.71 to -0.78). These results indicate that the rank of provenance cold hardiness determined by two different methods in two consecutive years could be similar. Due to unusually mild weather in autumn of 1999, trees started to acclimate later than in 2000. This is probably why cold hardiness in October in 1999 was significantly correlated with that in September in 2000.

In general, the degree of agreement between chlorophyll fluorescence and electrical conductivity or cambium visual scoring for detecting freezing damage was poor in comparison with other reports. Strong correlations between Fv/Fm and visual assessment have been observed for white spruce, Douglas-fir (Fisker *et al.*, 1995;

Hawkins and Lister, 1985), lodgepole pine and Scots pine (Lindgren and Hallgren, 1993). The low correlations between methods could have been caused by many reasons. In contrast to the above-mentioned studies, in which homogeneous needles of conifer were used as experimental materials, we collected samples from different sources and tissues of broadleaf species, and sites. Genetic control of stem and leaf cold hardiness development may have different regulatory controls as reported by Timmis (1976), Burr *et al.* (1990) and Aitken *et al.* (1996); tissues at different sampling position (leaves were collected from at the stem apex and stem tissues came from the middle of tree) might be at different physiological phases and cold hardiness levels (Timmis, 1976); the same provenance growing in different environments might show different acclimatization times or rates; and most importantly, on the cellular level, the Fv/Fm ratio measures photosynthetic activity in the chloroplast and electrical conductivity measures leakage of cell electrolytic contents from cell membrane disruption due to freezing. There is no known biological reason for the two cellular compartments to behave in a correlative fashion in respect to freezing tolerance as demonstrated by Devisscher *et al.* (1995) and Öquist and Strand (1986). Generally poorer correlations found in this study could have been caused by any one reason mentioned above or their combinations.

Adaptive Variation

In general, population differentiation occurs across environmental gradients. Earlier work has demonstrated that genetic variation for cold hardiness among provenances is strongly related to geographic variables (Lindgren and Hallgren, 1993;

Simpson, 1994) or climatic variables (Parker *et al.* 1994; Parker and van Niejenhuis, 1996). The genetic differentiation for cold hardiness, as an adaptive trait, is mainly controlled by climatic variables in northwestern Ontario (Parker *et al.* 1994; Parker and van Niejenhuis, 1996).

Cold hardiness estimated by Fv/Fm in September was significantly related to climate of each seed source except in the case of leaf Fv/Fm in 1999 (Table 4). However, in October no significant predicting equation was found. Similarly, the relationship between cold hardiness estimated by CVS and climatic variables was strong in September but not in October. Cold hardiness estimated by EC showed significant relationships with climate both in September and October. The r^2 values obtained between stem Fv/Fm and climate were comparable to those obtained both with leaf Fv/Fm, and CVS. Compared with CF and CVS, the r^2 values obtained in September between cold hardiness estimated by electrical conductivity and climate were relative lower in September but higher in October.

The independent variables retained in equations were not completely consistent between tissues, between the months of September and October, between years, and between methods. As discussed before, leaf and stem tissues may acclimate at different rate; trees growing in northwestern Ontario in September and in October are likely in different stages of cold hardiness development. Also, CF, EC and CVS detect different physiological responses to freezing. Thus the different climatic variables remaining in the models are indicators of different adaptive responses of different tissues, times or even physiological processes to cold stress.

CONCLUSIONS

CF, EC and CVS can be used to test cold hardiness changes in aspen. With the decrease in freezing test temperature, the damage detected by three methods was greater. Correlations between methods varied depending on tissue and sampling date. Some high correlations were obtained between stem freezing damage estimated by CF and CVS within the same year and between CF and EC in two consecutive years. As well, the high coefficients of determination with climate indicate that stem Fv/Fm could be a good method to detect genetic differentiation in cold hardiness in September.

Mainly due to low experimental errors, the percentage of variation expressed among provenances estimated by EC were higher than those of CF and CVS, indicating that the EC technique is a more sensitive test of genetic variation. Further research on the CF procedures and how environmental factors affect CF results is necessary.

Although the adaptive mechanisms detected by CF, EC or CVS might be different, any of these techniques could be a good method to detect genetic adaptive variation in September. However, in October, only the EC technique detected genetic adaptive variation in cold hardiness among aspen populations. The high genetic variation in cold hardiness of aspen populations obtained in September by three methods indicates that it is a suitable time to test genetic differentiation in cold hardiness.

PART II: GENETIC VARIATION IN COLD HARDINESS AMONG ASPEN PROVENANCES FROM NORTHWESTERN ONTARIO

INTRODUCTION

Cold hardiness, an important adaptive trait, is critical to the survival and productivity of plants. Cold hardiness has been successfully used to differentiate populations of many North American tree species. For example, large provenance differences in cold hardiness have been reported in eastern larch (Joyce, 1988), Douglas-fir (Larsen, 1978; Rehfeldt, 1979), Sitka spruce (Cannel and Sheppard, 1982), white spruce (Simpson, 1994) and jack pine (Parker and van Niejenenhuis, 1996). In most cases, differences among provenances are related to geographic variables or climatic variables (Joyce, 1988; Balduman *et al.* 1999; Simpson, 1994; Parker and van Niejenenhuis, 1996).

Aspen (*Populus tremuloides* Michx.) is widely distributed throughout Canada and can grow under a wide range of climate conditions. It constitutes more than 50% of Canada's deciduous timber volume and 12% of its total forest resources. However, because aspen has only recently become economically appealing to forestry industries in North America, little research has been done on its genetic variation. Most of the information on genetic variation in aspen has come from phenotypic observations (Greene, 1971; Barnes, 1975). Aspen provenance tests have only recently been started (W. Parker, personal communication, 1999). No information is available on differences in cold hardiness among aspen provenances.

As a part of a short-term research program on adaptive variation of aspen provenances, the objectives of this study were to describe the general cold hardiness development trend of aspen populations from northwest Ontario, to assess the pattern of adaptive variation in cold hardiness, and to gain some insight regarding aspen seed transfer in this region.

MATERIALS AND METHODS

One-year old aspen seedlings grown from 20 seed sources (1, 2, 3, 4, 5, 7, 8, 9, 10, 12, 14, 15, 17, 18, 19, 20, 23, 24, 25, 26) in northwestern Ontario were planted as border trees of aspen provenance test at Kreikmann (KRKM), Camp 45(C45) and Dog River (DR) in June, 1998 (Table 1 and Figure 1). Twenty trees of each origin were sampled in 1999: i) 5 September (KRKM), ii) 15 September (C45), iii) 25 September (DR), iv) 5 -7 October (KRKM, C45 and DR), and v) 5 November (KRKM). On each sampling date three twigs of the current-year growth were collected from the middle portion of each tree, sealed in a wet plastic bag and kept at 5°C in a walk-in freezer until freezing tests began. However, to avoid excessive damage to the seedlings, repetitive samples of the same provenance collected in September, October and November were not necessarily taken from the same trees. Each twig was cut into three segments approximately 1cm in length starting from the top of twig, and the segments from each origin were randomly divided among nine test vials, with three vials for each temperature.

While three vials were kept at 5°C as a control, the others were placed in a programmable freezer. Preliminary tests demonstrated that temperatures inside vials

were the same as air temperature inside the freezer, so only the air temperature inside freezer was monitored by several thermocouple thermometers during freezing. The treatment temperatures varied from -10°C to -50°C depending on the date (Table 7). These temperatures were selected based on preliminary tests and cold hardiness research on jack pine and black spruce in Northwestern Ontario (Parker and van Niejenhuis, 1996). After half an hour at +5°C, the temperature was reduced by 6°C per hour to one preset temperature and held for one hour. Then the temperature was increased by 10°C per hour to 5°C was reached and 20ml of deionized water was added to each vial. After 24 hours, the relative conductivity (EC), called EC(treated) and EC(control), were measured using the electrolyte leakage test (Colombo et al., 1984). Samples were then autoclaved at 110°C for one hour, and kept at 5°C for another 24 hours, at which time a third electrical conductivity EC(killed), was measured. Conductivity was measured with a YSI MODEL 35 CONDUCTANCE METER (Yellow Springs Instrument Co., Inc.). Frost hardiness was expressed as relative conductivity (RC) and index of injury (I_t), which are estimated by following formulae respectively:

$$RC_{(\text{control or treated})} = EC_{(\text{control or treated})} / EC_{(\text{killed})};$$

$$I_t = 100*(RC_{(\text{treated})} - RC_{(\text{control})}) / (1 - RC_{(\text{control})});$$

Index of injury is a better indicator of cold hardiness since it eliminates the release of electrolyte that causes without freezing. Thus most of analysis in this study focused on index of injury (I_t). A lower RC or I_t value represents a higher cold hardiness level.

Climate Data

The derivation of climate data is described in the materials and methods of PART I . A total of sixty three climate variables for each seed origin were used in this study. These variables include mean, minimum and maximum temperature for each month, monthly precipitation, and a number of growing season variables including length of the growing season and growing degree days for each growing period. Table 6 lists variables that had significant effects on genetic differentiation in cold hardiness among aspen populations from northwestern Ontario. Significant variation across the region exists within each variable.

Data Analysis

Data were subjected to analysis of variance, principal component analysis, and multiple linear regression. SAS programs (1989) were used for all data analysis. Inspection of residuals and variances indicated that all traits conformed well to assumptions of analysis of variance, thus untransformed variates were analyzed in all cases. Provenance effects were analyzed at each freezing temperature at each provenance trial site based on the model:

$$Y_{ij} = \mu + P_i + \varepsilon_{(i)j};$$

where Y_{ij} is one measure of I_t, μ is the estimate of the overall means of I_t, P_i is the random effect of the $_i$th provenance, and $\varepsilon_{(i)j}$ is the random effect of the $_j$th replicate.

Table 7. Summary of climatic data obtained from the digital Ontario Climatic Model for 20 aspen seed sources from northwestern Ontario

Prov.	FebMaxt(°C)	JunMaxt(°C)	AugMaxt(°C)	AugPrec(mm)	gdd2	mnt3(°C)
1	-6.7	21.3	22.8	88.8	245	13.3
2	-6.6	21.2	22.8	89.7	241	13.2
3	-7.1	21	22.5	89.8	245	13.1
4	-6.9	20.8	22.5	87.7	241	12.9
5	-7.1	20.6	22.4	87.1	240	12.8
7	-6.3	20.2	22.6	84.9	226	13.1
8	-8.1	20.8	22.2	86.6	255	13
9	-8	20.8	22.2	86	257	13
10	-7.9	20.8	22.3	85	251	13
12	-6.9	20.9	22.7	83.3	238	13
14	-7.7	19.3	21.5	91.8	227	12.6
15	-8.4	20.1	21.8	87.1	244	13
17	-9	20.2	21.9	88.4	248	13
18	-8.6	20.3	22.2	86.8	249	13.1
19	-8.7	20.3	22	87.3	246	13
20	-7.4	19.9	21.9	87.9	230	12.8
23	-7.4	19.6	22.1	91.2	233	12.8
24	-7.7	19.6	21.6	89.9	231	12.7
25	-7.1	19.1	21.8	87.4	230	12.7
26	-7.6	19.1	21.6	89.8	222	12.5
means	-7.56	20.29	22.17	87.82	239.95	12.93
CV	-9.88	3.35	1.82	2.46	4.17	1.57

Note: Febmaxt, maximum temperature in February; Junmaxt, maximum temperature in June; Augmaxt, maximum temperature in August; Augprec, month precipitation in August; gdd2, growing degree days of period 2---the first six weeks after starting growth (heat sum>5°); mnt3, mean temperature for period 3---entire growing season.

Variance components were estimated using the restricted maximum likelihood (REML) estimator.

In order to summarize variation in cold hardiness and analyze the relationship between variation and climate variables, principal components analysis (PCA), which reduces many correlated variables to a few meaningful uncorrelated components, was used to generate significant principal components (PCs). These PCs were then regressed against climate variables unique to each seed source to indicate the adaptive variation pattern. The 'maxr' option of SAS was used to determine significant ($p < 0.05$) regressions based on the best five-variable combination of climate factors.

RESULTS

Test temperatures were chosen to inflict, on average, intermediate levels of tissue damage (i.e., with mean RC around 0.50). The expected damage levels were achieved in September treatments for KRKM and Camp 45, but not in other cases. Thus the critical temperatures (e.g., the temperatures at which RC reached 0.50) were not estimated in this study.

Cold hardiness development expressed by RC and I_t were similar. Under each test combination, RC and I_t increased with decreasing test temperatures (Table 7). However, the index of injury (I_t) is a better indicator because it allows the direct comparison of cold hardiness level between test temperatures, between sampling sites or between sampling dates directly. The I_t varied with sampling date and site. At KRKM, the index of injury decreased from 48.49% on September 5 to 3.29 % on October 5 at

Table 8: Relative conductivity (RC) means(standard deviation), mean index of injury (I_t) and percentage of variation expressed among provenance by I_t by freezing in 1999

SITE	DATE	TEMP(°C)	RC(SD)	I_t(SD)(%)	r_I[a]
KRKM	SEPT. 5, '99	5	0.316 (0.03)	-	-
		-10	0.493 (0.06)	28.10 (3.60)	79.57**
		-20	0.645 (0.05)	48.49 (6.01)	60.93**
	OCT. 2, '99	5	0.239 (0.04)	-	-
		-20	0.267 (0.04)	3.29 (3.82)	74.80**
		-40	0.293 (0.03)	13.48 (3.74)	81.72**
	NOV.1, '99	5	0.227 (0.03)	-	-
		-30	0.251 (0.02)	3.26 (2.06)	41.79*
		-50	0.255 (0.03)	5.14 (3.15)	48.83*
CAMP 45	SEPT.15, '99	5	0.245 (0.02)	-	-
		-10	0.471 (0.07)	31.19 (4.91)	74.85**
		-20	0.586 (0.07)	46.47 (5.53)	82.68**
	OCT. 3, '99	5	0.225 (0.03)	-	-
		-20	0.306 (0.04)	10.19 (4.78)	72.09**
		-40	0.365 (0.04)	18.44 (3.69)	67.03**
DR	SEPT. 25, '99	5	0.196 (0.08)	-	-
		-10	0.237 (0.08)	4.84 (2.26)	73.54**
		-20	0.366 (0.08)	20.68 (4.50)	87.52**
	OCT. 4	5	0.207 (0.05)	-	-
		-20	0.246(0.05)	5.46 (2.40)	42.33*
		-40	0.315(0.05)	13.60 (2.62)	81.10**

[a] r_I, intraclass correlation coefficient.
*significant at 0.05.
** significant at 0.01.

–20°C, and further reduced to 5.14% at -50°C on November 1. Results for the other two sites showed similar hardiness development trends, but the later starting dates together with no sampling in November resulted in a decrease in the range of injury. The significant reduction of index of injury from September to October suggests that cold hardiness became well developed over the two to three weeks of late September.

Provenances growing at different sites showed different rates of cold hardiness development. The similar index of injury values for the same temperatures between KRKM samples and C45 samples in the September treatments indicate that cold hardiness development was about 10 days later at C45 than at KRKM. Direct comparisons of I_t under the same test temperatures in October suggest that cold hardiness progressed more rapidly at KRKM and DR compared to Camp 45.

Significant provenance effects on stem cold hardiness were found for all treatments (Table 7). For most of experimental combinations, the provenance component accounted for over 70% of total variation. The high variation among provenances suggests that aspen is a highly genetically differentiated species in terms of cold hardiness in this area. The percent variance expressed among provenances varied with sampling date. Slightly higher variation among provenances for each site appeared in September, followed by October and November. Variance components also varied with test temperature, but no consistent pattern of variance component size corresponding to temperature was observed. Despite the differences in cold hardiness, variation among populations was comparable among sites in early October, and ranged from 70% to 80%.

Principal component analysis of the 14 selected variables showed that 78% of

the sample variation was accounted for by just four PCA axes, indicating that strong correlations were present among the variables (Table 8). PC-1, PC-2 and PC-3 accounted for 68% of the variation over all 14 traits. PC-4 accounted for less than 10% of the total variation among traits and represents combinations of traits that are difficult to interpret biologically. PC-4 will not be discussed further.

All variable loadings were positive for PC-1 (Table 8). Eigenvector loadings for PC-1, varied from an absolute value of 0.03 to a relatively high value of 0.38. These loadings increased in magnitude with the development of cold hardiness from late September to early November, suggesting that PC-1 seems to reflect cold hardiness development in October and November. Due to the late sampling date (25 September), the loading for DR-Sept.-20°C also was high. Since the eigenvector loadings for all October and November experiments were positive and strong, strong positive genetic correlations may exist for cold hardiness among test sites, and between cold hardiness development in October and November.

Provenances with larger scores for PC-1(see Appendix D) tended to have relatively high degrees of cold injury in October and November. Figure 2 is a three-dimensional distribution of provenance scores for PC-1, PC-2 and PC-3. Provenances with higher PC-1 scores possessed relatively poorer cold resistance. It is notable that a weak southwest to east cline in cold hardiness existed for aspen populations in this region. Provenances from the southwest of the study area, such as 1, 3, 4 and 8, had a higher degree of injury while provenances from the eastern part, including 25, 26 and 15, exhibited a relatively higher level of cold hardiness. However, many inconsistencies were evident. For example, provenance #18, a northern source, exhibited the lowest

Table 9: Results of principal components analysis of 14 freezing variables (index of injury) for 20 provenances

	Eigenvalue	Difference	Proportion	Cumulative
1	5.08351416	2.60198865	0.3631	0.3631
2	2.48152551	0.52038261	0.1773	0.5404
3	1.96114290	0.63165715	0.1401	0.6804
4	1.32948575		0.0950	0.7754

Eigenvectors

	Prin1	Prin2	Prin3	Prin4
KRKM-Sept-10°C	0.065145	0.235410	-.356657	0.364654
KRKM-Sept-20°C	0.131079	0.335407	0.069018	-.131667
C45-Sept-10°C	0.033796	0.166074	0.546964	0.093882
C45-Sept-20°C	0.184743	0.345319	0.433780	-.096719
DR-Sept-10°C	0.164219	0.489971	-.108215	-.024874
DR-Sept-20°C	0.295819	0.228400	-.268005	0.336767
KRKM-Oct-20°C	0.280130	-.253539	0.257400	0.440044
KRKM-Oct-40°C	0.290654	-.115229	0.361273	0.359980
C45-Oct-20°C	0.334785	-.243800	-.014898	-.394640
C45-Oct-40°C	0.283152	-.128470	0.111494	-.434202
DR-Oct-20°C	0.310178	0.299302	-.123387	-.193576
DR-Oct-40°C	0.386756	0.068382	-.076470	-.054208
KRKM-Nov-30°C	0.313838	-.325314	-.171783	0.094733
KRKM-Nov-50°C	0.359627	-.198570	-.192750	-.024736

[a] Freezing trial sites, dates and temperatures: KRKM-Sept.-10°C: kreikmann, 5 September and -10°C treatment; KRKM-Sept.-20°C: kreikmann, 5 September and -20°C treatment; C45-Sept.-10°C: Camp 45, 15 September and -10°C treatment; C45-Sept.-20°C: Camp 45, 15 September and -20°C treatment; DR-Sept.-10°C: DR, 25 September and -10°C treatment; DR-Sept.--20°C: DR, 25 September and -20°C treatment; KRKM-Oct.-20°C: kreikmann , 5 October and -20°C treatment; KRKM-Oct.-40°C: kreikmann , 5 October and -40°C treatment; C45-Oct.-20°C: Camp 45, 6 October and -20°C treatment; C45-Oct.-40°C: Camp 45, 6 October and -40°C treatment; DR-Oct.-20°C: DR, 7 October and -20°C treatment; DR-Oct.-40°C: Dog river, 7 October and -40°C treatment; KRKM-Nov.-30°C: kreikmann, 2 November and -30°C treatment; KRKM-Nov.-50°C: kreikmann, 2 November and -50°C treatment.

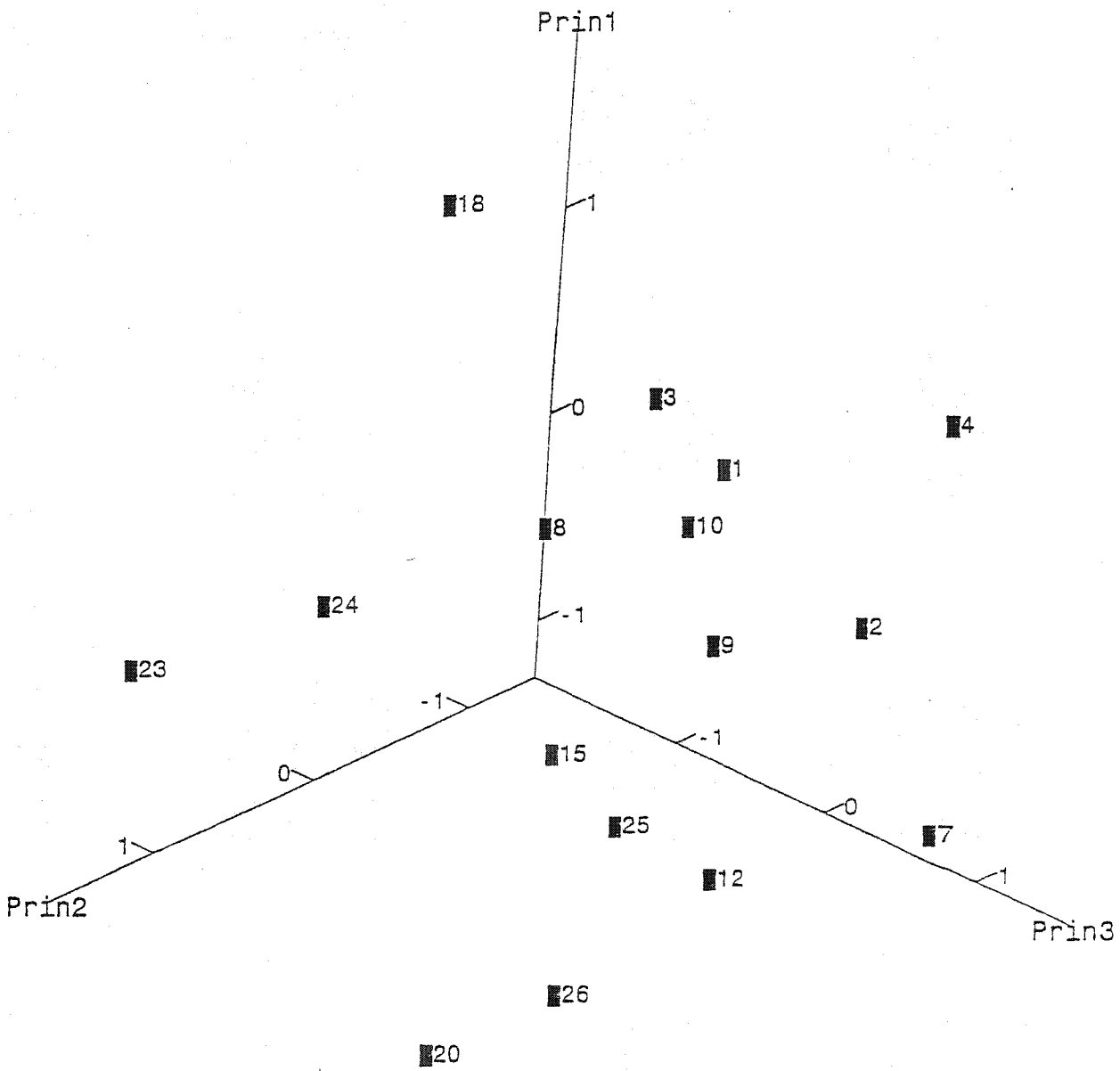


Figure 2. Three-dimension distribution of scores for PC-1, PC-2 and PC-3 of each provenance. Provenance #5, #14, #17 and #19 were only tested at one test temperature, thus the scores for these provenances could not be determined.

level of frost hardiness among all provenances.

Eigenvector loadings for PC-2, varied from an absolute value 0.06 to relatively higher value of 0.49. These loadings were all positive and strong for September variables but negative and weak for October and November variables except DR-Oct-20°C and DR-Oct-40°C. These values have two implications, first, PC-2 largely reflects cold hardiness development in early fall; second, the correlation between September variables and October or November variables was poor and acclimatization rate of the provenances was different for early and late stages of cold hardiness development. Provenances with larger scores for PC-2 (see Appendix D) tend to have relative high degree of injury in early September (Figure 2). In contrast with PC-1, a clear eastern to western trend in cold hardiness was found. Provenances from the eastern part of study area, such as provenances 20, 23, 24 and 26, showed poor resistance, while provenances from the northwestern portion of the study area, such as provenance 1, 2, 3 and 4, indicated high resistance to low temperature in early fall.

PC-3 mainly explains the variation of September cold hardiness development at C45 (Table 8). However, DR-Sept-20°C, KRKM-Oct-20°C and KRKM-Oct-40°C also make large contributions to PC-3 with mixed sign. No significant geographic trend was noted. Provenance 20, 26 and 7 showed relatively higher frost damage, while provenances 18, 23, 8 and 24 indicated higher cold resistance in September at site Camp 45.

Table 9 shows the results of simple regression using three principal components (PC1, PC2 and PC3) against each climate

variable unique to each source. Many good predictive regression models were obtained,

Table 10: Multiple regression analysis results between PC-1, PC-2 and PC-3 with climatic variables unique to each seed source from northwestern Ontario

Variable	Independent variables*	r^2	Pr>F
PC-1	GDD2, AugPrec	0.38	0.0464
PC-2	JunMaxt , AugMaxt, Mnt3	0.53	0.0239
PC-3	FebMaxt	0.34	0.017

* Febmaxt, maximum temperature in February; Junmaxt, maximum temperature in June; Augmaxt, maximum temperature in August; Augprec, month precipitation in August; gdd2, growing degree days of period 2---the first six weeks after starting growth (heat sum>5°); mnt3, mean temperature for period 3---entire growing season.

but only the best one for each principal component are shown here based on their high r^2 and biological meaning. A relationship of PC-1 with growing degree days in the early period of the growing season and August mean precipitation was found (r^2=0.38). Sources from area dry in summer (low August mean precipitation) together with low growing degree days of during the first six weeks of the growing season (heat sum>5°) were predicted to have high cold hardiness in October and November. Significant regression models were found (r^2=0.53) for PC-2 against monthly maximum temperature in June and August, and mean temperature for the entire growing season. In this case, seed sources from areas with high mean temperature for the entire growing season, and high maximum temperature in June and August were predicted to have higher cold hardiness in September. The regression of PC-3 against February maximum temperature was also significant (r^2=0.34). Sources from areas with higher February

maximum temperatures were predicted to have poorer cold resistance.

DISCUSSION

Cold hardiness in hardy plants changes seasonally from late August to midwinter in temperate areas. According to Weiser (1970) and Glerum (1976), the cold hardening process, under natural conditions, proceeds in two phases in temperate woody plants. The first phase occurs in early fall and is associated with the cessation of growth. The second phase is induced by low temperature, and it is in this stage that large increases in cold hardiness occur. The results of this study partly support this theory. As expected, the rapid reduction in index of injury (I_t) from September to October indicated that cold hardiness of aspen developed cold hardiness rapid within two to three weeks from mid- to late September. These results, together with the results of principal components analysis, suggest that cold hardiness development of aspen could be divided into two phases. The first phase, mainly explained by PC-2 and PC-3, would likely be initiated between early and middle September. The second phase, characterized by fast reduction in index of injury and explained by PC-1, would be initiated from late September, and it is in this phase that cold hardiness is rapid developed. Other reports have argued that the acclimation process may start in August in temperate zones or the second phase may start in November (Sakai 1983), but we did not assess hardiness sufficiently early or late to test these hypotheses. Thus the conclusion should be treated with caution.

Cold hardiness changes noticeably in response to environment. Provenances develop cold hardiness at different rates under different environment stress. In early

October, index of injury was higher for Camp 45 experiments at both treatment temperatures, than those observed for DR and KRKM, suggesting that trees growing at Camp 45 possessed a lower cold hardiness level than those growing at KRKM and DR. The similar index of injury in September 5 experiments at KRKM and September 15 experiments at Camp 45 suggest that cold hardiness development in Camp 45 was about 10 days later than for KRKM. This developmental lag in cold hardiness was most probably due to the large differences in environment. Due to mild weather, trees had a relatively longer growing season at Camp 45. As described before, genetic variation in cold hardiness within woody species appears to result mostly from variation in starting time of cold hardening. The low level of cold hardiness of trees growing at Camp 45 is most likely due to late timing of cold hardening.

Aspen genotypes varied in cold hardiness according to their origin. Very high variance components expressed among provenances, in most cases over 70%, indicate that aspen in this region is genetically variable and highly differentiated. Percent variation expressed among provenances varied depending on sampling dates and test temperatures within sampling date. As reported for other tree species (Rehfeldt, 1979), slightly higher variation expressed among populations was observed in September, compared to October and November (Table 7), suggesting that genetic differences in fall cold hardiness among aspen populations were greatest in the first phase (September) of acclimation. The maximum hardiness attained by all provenances is likely great in mid-winter. However, the time when this level is achieved varies between provenances. Thus, testing in September turns out to be best. Variance components also changed with treatment temperature, most often being greater at lower temperature.

The loadings and scores for PC-1 and PC-2 suggest that the hardiness of aspen provenances during September was not related to hardiness during October and November. This disagreement might be a result of different acclimation timing or rate for individual provenances. It is notable that provenances from the eastern part of the study area started to acclimate early, but develop relatively slower in late September compared to those from the southern part of the study area. However, a lot of inconsistencies were present in the study.

Geographic variation in cold hardiness appears to be an adaptive response to environment, especially temperature, moisture and photoperiod. Since the first three principal component axes accounted for 68% of the total variation, their correspondence with climatic variables presumably reflects adaptive variation. Regression results in this study partly support this hypothesis. Regression models accounted for 38% and 53% of the variance for the first and second PC axes respectively. Growing season length and precipitation in August at the site of each seed origin were the best predictors of second phase, while temperature was always the best predicator of the first phase. Sources from summer dry areas with more heat during the growing season were less cold hardy in October and November. Sources from areas with dry summer showed higher cold hardiness levels in September. These results were partly in agreement with observations for other tree species in this region. For example, Parker et al. (1994, 1996) investigated the adaptive variation of black spruce populations from northwestern Ontario, and suggested that black spruce is a specialist in terms of its adaptive response to climate. The differentiation in cold hardiness among black spruce populations was reflected by temperature in January, growing degree days and precipitation (r^2=0.559). Davradou

(1992) found that extreme minimum temperatures, precipitation and mean daily temperature were the climatic gradient which most frequently appeared to be associated with cold hardiness of jack pine in northwestern Ontario.

Results in this study have two implications for aspen improvement. First, for a breeding program, it is necessary to determine patterns of genetic variation in the traits of interest, which indicate where selection pressure can be applied to obtain greatest benefit. High variation among populations means that selection of provenances will accrue large gains in cold hardiness. Second, although differentiation among aspen populations in this region does not strongly follow geographic gradients, as demonstrated in other tree species in this region (Davradou, 1992; Parker *et al.* 1994), this irregular performance was predicted by climatic variables that vary a great deal over fairly short geographic distances in the sampled portion of northwestern Ontario. Thus, special attention is required when seed transfers are contemplated in this region to avoid the establishment of maladapted source material that may result in decreased growth and survival due to inadequate cold hardiness.

PART Ⅲ: COLD HARDINESS OF ASPEN STEMS ASSESSED BY CHLOROPHYLL FLUORESCENCE

INTRODUCTION

The economic importance of aspen (*Populus tremuloides* Michx) mainly lies in its fast growth rate and high biomass productivity. Environmental stresses can however reduce, sometimes drastically, the biomass production of aspen. One of the best strategies to solve these problems is to use genetically improved material, such as provenances, clones or families, which are most successful in coping with these stresses.

Cold hardiness, an adaptation to low temperatures is crucial to the success of tree improvement programs in northwestern Ontario. The genetic variation in cold hardiness of important tree species in this region, such as jack pine and black spruce, has been well documented (Davradou, 1992; Parker and van Niejenhuis, 1996). For these species, significant genetic variation among populations and clinal trends following climatic gradients have been demonstrated in this region. The results of parts Ⅰ and Ⅱ of this study have shown that aspen populations from northwestern Ontario differed significantly in cold hardiness, and further suggested that provenance selection for improving cold hardiness could be effective.

While cold hardiness assessed by electrical conductivity and visual scoring techniques yield reliable results, each method has certain disadvantages. The visual scoring method is subjective and the results may differ between observers; the

appearance of injury generally requires an extended period of time (usually one week). Electrical conductivity is a very sensitive but time-consuming method. In breeding programs, the purpose is to rank hundreds or even thousands of genotypes. A simple, rapid and reliable method of ranking provenances, progeny or clones would be of considerable value.

In recent years, chlorophyll fluorescence has been widely used in studies to determine how environmental factors including freezing, alter the photosynthetic capacity of plants. Using needles as test material, this technique has been successfully used to assess cold hardiness development in evergreen conifer tree species, including white spruce (Binder and Fielder, 1996), Douglas-fir (Hawkins and Lister, 1985; Fisker *et al.*, 1995), black spruce (Devisscher *et al.*, 1995), Scots pine (Sundblad, 1990), lodgepole pine and Scots pine (Lindgren and Hallgren, 1993). High correlations between chlorophyll fluorescence and visual assessment or electrolyte leakage tests of damage were demonstrated in those experiments.

In the above-mentioned experiments, needles from evergreen conifers were used as samples. For deciduous tree species like aspen, it is difficult to collect non-senescent leaves in middle to late September, a critical period to test genetic differentiation in cold hardiness in northwestern Ontario. In studies of bilberry and willow stems, Ögren (1996, 1998) found a high correlation between cold hardiness assessed by chlorophyll fluorescence, electrical conductivity or visual scoring and concluded that chlorophyll fluorescence method is applicable to stems provided that the green inner bark can be assessed through the outer bark.

The purpose of this study was to quantify the degree of genetic variation among

34 aspen seed sources and three hybrids in fall cold hardiness using chlorophyll fluorescence of detached stems. The results were compared with those obtained by a traditional method of visual cambium scoring injury after freezing.

MATERIALS AND METHODS

One-year old aspen seedlings from 37 locations (Table 1) were planted as a provenance test at Camp 45(C45) in June of 1998. Twenty six of these sources were collected from northwestern Ontario by Lakehead University; one source was provided by Weyerhaeuser from Alberta; and ten additional sources were provided by the Aspen-Larch Genetics Cooperative including: three from Alberta and Saskatchewan, one from Minnesota, two European sources of *Populus tremula* L. and three full sib hybrid families of *P.tremula* x *P. tremuloides*. Twenty trees of each origin were sampled on 15 September and 1 October, 2000. On each sampling date, three healthy twigs, five centimeters in length, of the current-year growth were collected from the middle part of each tree, sealed in wet plastic bag, transported to the laboratory and kept at 5°C overnight. The freezing treatments were carried out the next day. Twigs from each origin were equally divided into 4 paper bags, one for each test temperature. Controls remained at 5°C, while the other bags were placed in a programmable freezer. Several thermocouple thermometers were used to monitor the temperature inside the freezer. After half an hour at 5°C, the temperature was lowered 6°C per hour to one preset test temperature and held for one hour. Then the temperature was increased by 10°C per hour to 5°C. After samples were removed from the freezer, the samples including controls were kept in a dark room for 2 hours (+10°C) prior to damage assessment. In

September, four freezing treatments were applied: +5°C (control), -10°C, -15°C and -20°C. In October the treatments were +5°C (control), -12°C, -22°C and -33°C. Damage caused by low temperature treatments was evaluated by chlorophyll fluorescence and cambium visual scoring. Due to limited samples, provenances 29, 32, 33 and 34 in September experiments, and 32, 33 and 34 in October experiments were only tested at one or two test temperatures.

Chlorophyll Fluorescence

Freezing injury to the photosynthetic tissue of the inner bark was quantified by probing chlorophyll fluorescence of the stem samples after they were dark-adapted for 2 hours at 10°C. A FMS-2 Fluorescence Monitoring System (Hansatech, England) was used. During measurement, the probe was held against the upper part (just below apical bud) of the twig and then exposed to a blue excitation beam of 3000 $\mu mol/m^2/s$. The fluorescence parameter Fv/Fm, the ratio of variable fluorescence (Fv) to maximum fluorescence (Fm), was recorded because other authors have demonstrated this to be a good indictor of frost hardiness (Fisker et al. 1991; Lindgren and Hallgren, 1993; Ögren 1996, 1999). Fv is the difference of the Fm and the nonvariable fluorescence characteristic of open reaction centers. Because Fv/Fm declines with the minimum test temperature, a percent reduction of Fv/Fm relative to the control samples is reported. A higher percent reduction in Fv/Fm suggests lower frost resistance.

Visual Scoring

After measurement of chlorophyll fluorescence, stems were put into vials with one vial for each provenance. 30ml of water was added to each vial and these vials were kept at room temperature (20°C) for one week. Water was changed every two days. Stem damage was examined by slicing stem lengthwise with a razor blade and measuring the length of discolored stem tissue (xylem/cambium). The percentage of the length of discolored stem to total stem length (5cm in length) was estimated to indicate freezing damage. A high percent damage indicates a relatively low cold hardiness level. Visual scoring was conducted only for sampling in October.

Data Analysis

SAS programs were used for all data analysis. Provenance effects at each temperature by each assessment method were analyzed whenever possible by the following linear model:

$$Y_{ij} = \mu + P_i + \varepsilon_{(i)j};$$

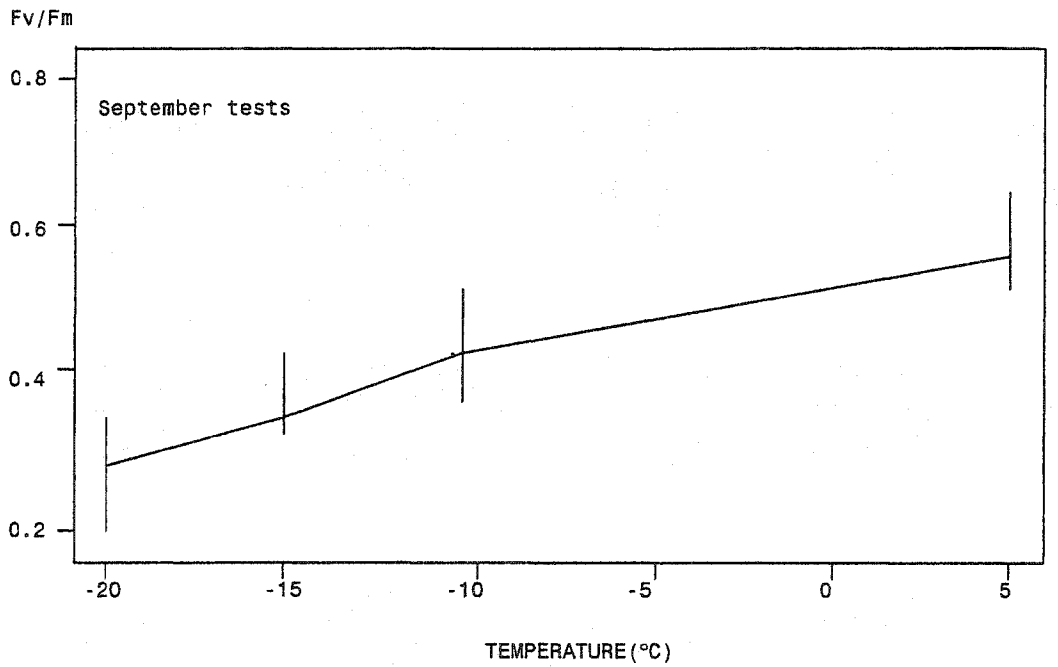
where Y_{ij} is the percent reduction of Fv/Fm relative to the control samples or visual damage individual value, μ is the mean of reduction in Fv/Fm (relative to the control samples) or visual damage, P_i is the random effect of the $_i$th provenance, and $\varepsilon_{(i)j}$ is the effect of the $_j$th replicate. The variance components for each factor were estimated by the restricted maximum likelihood (REML).

To evaluate the relationship between the assessment methods, simple correlation

was run between percent reduction of Fv/Fm and percent visual damage. Because decreasing test temperatures caused a linear reduction in Fv/Fm and corresponding linear increase in cambium visual damage, the temperature which caused a 50% reduction of Fv/Fm or 20% of visual damage could be estimated by a linear model for each provenance. Thus, temperatures which resulted in a 20% cambium damage and 50% reduction of Fv/Fm were estimated respectively. The correlation between those two critical temperatures was further analyzed by simple correlation analysis.

RESULTS

Freezing reduced Fv/Fm in all provenances and hybrids (Figure 3) In September, the average Fv/Fm ratio across provenances decreased from 0.56 at 5°C to 0.29 at -20°C, while in October it decreased from 0.52 at 5°C to 0.33 at -33°C. Although the reducing range was different for each provenance, the -10°C, -15°C and -20°C treatments in September caused an average of 21.5%, 35.2% and 49.1% reduction of Fv/Fm, while -10°C, -22°C and -33°C treatments in October resulted in average reduction of 8.6%, 24% and 37.2% decreases, respectively (Table 10). The lower Fv/Fm ratio reduction relative to control samples in October under the same (-10°C) or similar temperatures (-20°C and -22°C) suggests that trees had become hardier in October than in September. The average temperature which caused a 50% reduction in Fv/Fm ratio relative to control samples decreased from –21.8°C (Crit_T1) in September to –45.°C (Crit_T2) in October (Table 10).



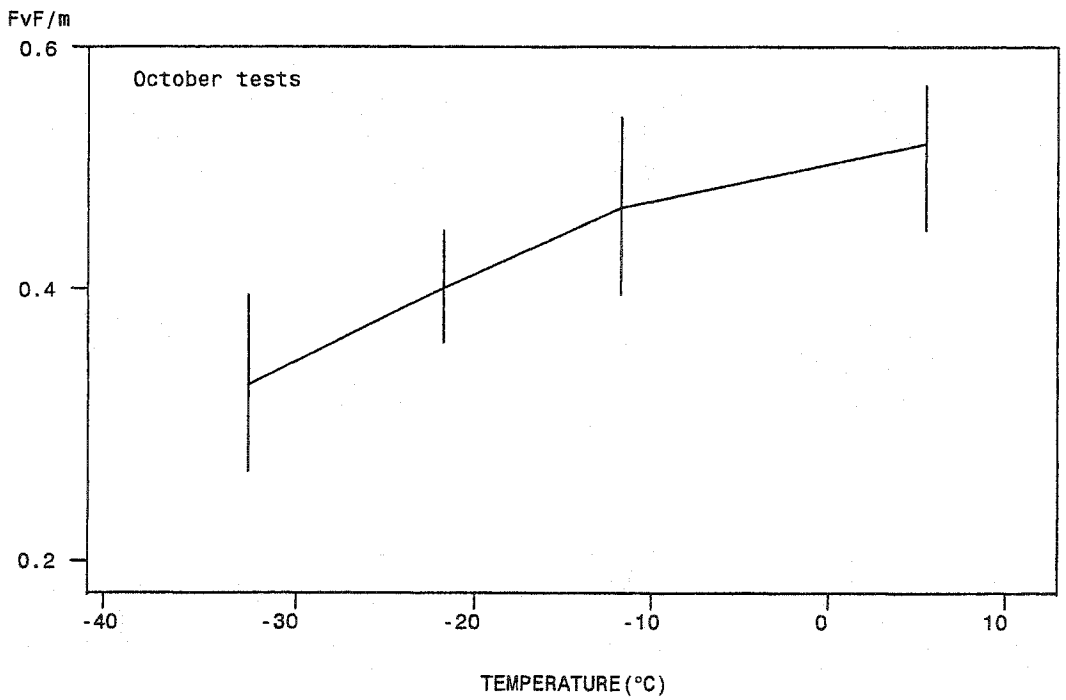


Figure 3. The mean Fv/Fm ratio and standard deviation across provenances at each test temperature

Table 11. Provenance means of percent reduction in Fv/Fm ratio relative to the control, percent damage of CVS, and critical temperatures

Method	CF								VS			
Date	Sept. 15, 2000				Oct.1, 2000				Oct.1, 2000			
P T	-10	-15	-20	Crit_T1	-10	-22	-33	Crit_T2	-10	-22	-33	Crit_T3
1	14.7	48.1	64.8	-16.52	12.4	27.0	36.2	-45.46	0	8.8	23.6	-30.72
2	22.4	49.7	62.2	-16.31	13.2	20.0	38.3	-45.96	0	19.6	44.8	-20.94
3	23.6	40.1	57.1	-17.90	7.9	23.0	35.9	-44.36	0	12.4	24.0	-29.29
4	31.3	35.2	60.2	-17.15	3.7	18.1	24.9	-58.60	0	8.4	20.0	-35.64
5	39.9	46.2	62.0	-15.28	2.4	18.2	24.3	-58.10	0	8.8	22.4	-30.93
6	21.3	38.0	47.4	-20.52	13.9	24.3	34.3	-61.75	0	14.0	26.4	-35.31
7	23.3	43.0	66.8	-16.29	3.8	16.1	28.1	-53.66	0	8.4	31.2	-26.65
8	25.0	43.4	69.0	-15.95	7.1	22.3	36.6	-43.57	0	12.8	36.0	-24.02
9	20.1	46.5	57.5	-17.44	9.9	15.5	33.2	-52.24	0	5.8	22.8	-32.42
10	18.8	37.8	61.7	-17.46	7.1	23.4	37.4	-42.36	0	19.6	31.6	-23.76
11	11.6	37.4	44.5	-20.72	3.6	18.5	30.7	-49.11	0	7.8	20.0	-33.90
12	27.9	34.7	45.4	-23.00	12.5	17.2	39.8	-44.69	0	12.0	17.2	-35.40
13	22.7	35.5	44.3	-22.32	2.8	16.0	20.1	-61.26	0	5.6	15.2	-41.39
14	29.5	36.4	53.6	-19.21	9.9	19.3	39.4	-42.78	3.6	9.2	20.0	-34.42
15	17.0	29.5	39.5	-24.48	16.7	30.2	51.4	-33.20	3.2	23.2	40.0	-20.35
16	25.9	35.1	57.8	-14.89	12.8	28.2	41.2	-39.81	0	12.4	20.0	-32.28
17	18.0	31.4	38.8	-24.90	12.9	22.6	40.2	-42.67	0	15.8	46.0	-21.36
18	20.5	46.3	62.7	-16.61	12.0	23.3	38.0	-44.24	2	16.8	42.0	-21.53
19	18.6	41.4	55.1	-18.64	11.4	25.4	33.4	-49.36	5.2	5.6	22.4	-33.64
20	7.1	26.4	37.7	-23.58	-2.0	18.0	40.1	-38.74	4.4	12.4	40.4	-22.29
21	15.1	29.9	35.7	-26.21	5.1	26.2	48.0	-34.37	0	3.2	28.0	-29.72
22	22.4	32.8	67.7	-16.99	3.5	23.3	28.8	-49.98	0	10.8	19.6	-33.34
23	26.8	33.5	43.5	-24.22	6.6	13.5	26.9	-60.63	0	5.6	16.4	-39.49
24	19.6	31.7	39.1	-25.18	1.9	20.4	31.0	-47.03	3.2	17.2	20.0	-30.40
25	20.9	28.8	54.1	-19.63	5.3	20.0	33.0	-46.83	0	14.8	18.4	-32.60
26	12.1	18.8	43.6	-22.98	11.4	26.5	32.7	-50.15	0	12.0	14.8	-38.93
27	28.1	38.3	47.6	-21.17	5.0	40.7	62.4	-27.28	0	15.6	46.8	-21.31
28	27.3	34.3	39.2	-28.78	12.5	29.7	45.9	-35.92	10	10.4	45.2	-20.39
29	29.0	.	.	.	13.0	26.4	33.0	-51.48	0	24.4	25.2	-24.78
30	20.3	30.5	43.2	-23.15	8.4	43.4	57.0	-28.06	0	23.6	47.6	-19.84
31	20.5	27.1	40.4	-25.39	0.6	36.4	56.2	-29.42	10.8	39.6	56.0	-13.83
32	2.1	.	17.2	.	4.7	.	30.9	.	0	.	35.2	.
33	17.6	26.3	.	.	4.6	.	.	.	0	.	.	.
34	37.6	.	46.2	.	11.6	.	.	.	0	.	.	.
35	28.7	32.3	44.3	-24.55	25.8	31.5	36.8	-29.41	0	17.2	25.6	-26.74
36	12.8	.	45.7	.	16.5	17.5	39.4	-47.76	0	14.4	21.6	-30.24
37	14.0	32.3	43.7	-21.73	8.0	33.3	37.2	-40.32	6.8	22.0	24.8	-24.36
mean	21.46	35.72	49.12	-21.76	8.6	23.98	37.22	-45.02	2.4	13.83	28.89	-28.60
r_I^*	23.36	23.53	37.5	/	0	29.36	23.15	/	0	8.53	22.29	/

* r_I , intraclass correlation coefficient.

Significant provenance effects were found in all freezing tests except -10°C in October (Table 10). The percent variance expressed among provenances was about 23% at -10°C and -15°C treatment, but increased to 37.5% at -20°C in September. In October, the highest percent variance expressed among provenances, 29%, appeared at -22°C treatment, and then reduced to 23% at -33°C treatment. It is notable that variation expressed among provenances was relatively lower in October than in September at the similar test temperatures (-20°C and -22°C). A temperature of -10°C was not low enough to differentiate genetic variation among provenances in October.

No significant geographic pattern in cold hardiness was found among aspen sources and hybrids. Sources provided by Larch-aspen CO-op had a relatively less cold damage in September, with critical temperatures of -24°C and -23°C for *P. tremula* and hybrid respectively, than most of the northwestern Ontario provenances. However, in October, the sources provided by Larch-aspen CO-op showed relative poorer cold resistance than most provenances from Ontario. The critical temperature which caused 50% reduction of Fv/Fm was higher than –30°C for *P. tremula* and –40°C for other sources from CO-op except 29(from Alberta) and 36(a hybrid from Michigan). In contrast to the results in Part II, no clear pattern in cold hardiness was indicated among the 26 northwestern Ontario provenances.

Similar results were obtained by the CVS method. Cambium visual damage increased with lower test temperatures (Table 10). At -10°C, -22°C and -33°C in October, there was an average of 2.4%, 13.8% and 28.9% cambium damage, respectively. As was true for CF results, significant provenance effects were demonstrated in all cases by CVS except for the -10°C treatment in October; unlike CF,

the variance expressed among populations increased with decreasing test temperature, ranging from <1% at -10°C to 22.3% at -33°C treatment (Table 10). Compared with the CF, variance expressed among provenances was much lower at -22°C treatment, only 8.5%, but similar at -33°C treatment, suggesting that photosynthetic tissues were more sensitive to freezing than cambium tissues. As was the case for CF, provenances provided by Larch-aspen Co-op and hybrids were less cold harden than most northwestern Ontario provenances in October.

Correlations between methods, sampling dates and temperatures within sampling date are listed in Table 11. Correlations between test temperatures below -10°C within sampling date were high (along diagonal) for both methods, from 0.68 to 0.79. Correlations between September and October experiments were generally negative. Significant positive correlations between percent Fv/Fm reduction and percentage visual cambium damage were found for the two colder test temperatures in October. The r value ranged from 0.52 to 0.73 for these treatments. The poor correlations observed between -10°C and the other temperatures in October were mainly due to the fact that -10°C was insufficient to induce damage to the photosynthetic system or cause cambium visual damage by the sample dates in October.

When cold hardiness was expressed bas a critical temperature, similar correlation relationships were demonstrated (Table 11). A significant correlation (r=0.72) was found between critical temperatures estimated by CF and CVS in the October trials. Regression analysis of the critical temperatures which resulted in 50% reduction of Fv/Fm versus the temperatures which causing 20% cambium visual

Table 12. Pearson correlation coefficients and their significance levels estimated from average percent Fv/Fm ratio reduction, percent damage of CVS, critical temperatures which resulted in 50% reduction in Fv/Fm ratio or caused 20% of cambium visual scoring among 37 aspen seed sources.

Date			Sept.15,1999			Oct.1,2000							
	Method		CF			CF				CVS			
		T(°C)	-15	-20	Crit_T1	-10	-22	-33	Crit_T2	-10	-22	-33	Crit_T3
Sept. 15 1999	CF	-10	0.36	0.44	0.26	0.15	-0.05	-0.09	-0.18	-0.1	-0.19	-0.23	-0.11
			0.041	0.007	0.157	0.384	0.793	0.608	0.318	0.57	0.249	0.177	0.563
		-15		0.71	0.69	0.14	-0.28	-0.24	-0.3	-0.23	-0.23	-0.03	0.02
				<0.0001	<0.0001	0.44	0.122	0.185	0.091	0.21	0.196	0.866	0.907
		-20			0.93	0.02	-0.27	-0.27	-0.35	-0.27	-0.22	-0.19	-0.11
					<0.0001	0.927	0.134	0.121	0.044	0.119	0.228	0.283	0.548
		Crit_T1				-0.07	-0.3	-0.39	-0.4	-0.46	-0.23	-0.25	-0.19
						0.704	0.093	0.026	0.022	0.008	0.201	0.164	0.292
Oct.1 2000	CF	-10					0.2	0.17	0.19	-0.12	0.11	0.03	0.06
							0.261	0.344	0.282	0.48	0.553	0.878	0.75
		-22						0.77	0.76	0.33	0.59	0.52	0.58
								<0.0001	<0.001	0.05	0.0003	0.002	0.0003
		-33							0.88	0.04	0.53	0.73	0.7
									<0.0001	0.81	0.001	<.0001	<0.0001
		Crit_T2								0.37	0.53	0.64	0.72
										0.03	0.001	<0.0001	<0.0001
	CVS	-10									0.16	0.17	0.45
											0.367	0.329	0.0078
		-22										0.68	0.68
												<.0001	<0.0001
		-33											0.79
													<0.0001

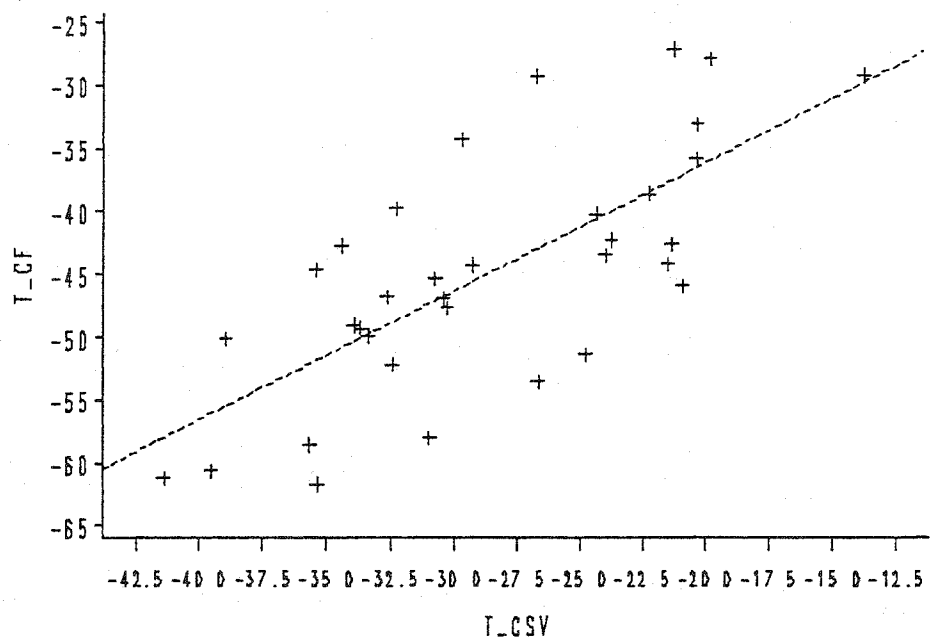


Figure 4. Regression between the critical temperatures which resulted in 50% reduction in Fv/Fm ratio (T_CF) and the critical temperatures which resulted in 20% cambium visual damage (T_CSV)

scoring in October indicated that over 50% of the variation expressed by Fv/Fm could be predicted by cambium visual damage (Figure 4).

DISCUSSION

On the cellular level, Fv/Fm measures photosynthetic activity. The ratio of variable fluorescence to maximum fluorescence (Fv/Fm) describes the photochemical efficiency of photosystem II. In this study, Fv/Fm progressively decreased with decreasing freezing temperatures, indicating that lower temperatures caused more damage to photosynthetic activity. Similar results were obtained in cold stressed

bilberry and willow stems by Ögren (1996, 1999), who demonstrated a linear relationship between Fv/Fm and freezing temperatures. Wulff et al. (1994) detected a progressive, often significant, decrease in Fv/Fm of Sitka spruce needles under decreasing temperatures. However, this result contradicts the observations of Adams and Perkins (1993). They found that there was no significant decrease in Fv/Fm of *Picea* needles until a critical freezing temperature was reached, which caused an abrupt reduction in chlorophyll fluorescence.

Some freezing damage measured by chlorophyll fluorescence is reversible (Strand and Öquist, 1988; Ögren, 1996; 1999), and as a result, period of incubation is important to precisely predict cold hardiness using this method. Ögren (1996, 1999) found that one week of incubation was needed before all damage was expressed. However, in our preliminary tests, no significant difference was found between Fv/Fm measured immediately after freezing and after one week (data not shown). The reason for this difference may reflect species specific or procedural differences. Because chlorophyll fluorescence is an indicator of photosynthetic system integrity, immediate reduction of Fv/Fm ratio after low temperature treatment, as well as no significant delayed reduction of Fv/Fm ratio of stems stored at 10°C, suggest that the photosynthetic system may be the initial site of freezing injury.

Significant variation in cold hardiness was found among provenances except for the -10°C treatment in October (Table 10), suggesting that aspen is a highly differentiated species in this region. Although there were some exceptions, most of the provenances from northwestern Ontario had lower cold hardiness in September but higher in October than provenances of *P. tremuloides* from other regions, as well as the

sources of *P. tremula* and their hybrids. This result might be caused by differences in the timing and rates of acclimation among provenances. Maybe, this is a kind of adaptive variation; trees from local seed sources stop growing later, and develop cold hardiness faster in late September. These results have implications in tree breeding programs involving improved cold hardiness.

Similar freezing results were obtained by the CVS and CF method. However, unlike CF, in which the highest variation expressed among provenances occurred for the –20°C treatment in September and October, the percent variance expressed among provenances increased with decreasing temperature in October. These results suggest that different tissues have different responses to the same freezing temperature and that photosynthetic tissue of the inner bark, measured in the CF method, was more sensitive than the cambium, measured using CVS, at -22°C.

One important objective in a breeding program is to rank genetic entities according to adaptive traits. Results in this study showed that chlorophyll fluorescence could be a valuable method to rank stem cold hardiness among provenances since it is simple, fast and accurate. High correlations between percent reduction of Fv/Fm and percent cambium visual damage suggest that the ranking of provenances by these methods could be similar as long as determinations are made in October. Chlorophyll fluorescence is rarely used to assess cold hardiness to stems. Ögren (1999) demonstrated a good agreement between cold hardiness of willow stems assessed by chlorophyll fluorescence and scoring of visible cambium discoloration, and further concluded that chlorophyll fluorescence is applicable to stems provided that the green inner bark can be detected through the outer bark.

The cold hardiness level for each provenance detected by CF and CVS was also expressed as the temperature resulting in a 50% reduction of Fv/Fm or 20% cambium damage. Critical temperature determinations allowed comparisons to be made between provenances, and it allowed further exploration of the relationships between cold hardiness variation and geographic and climatic variables. Several valuable methods have been used to derive critical temperature values related to the chlorophyll fluorescence technique. Adams and Perkins (1993) based their critical temperature values on the temperature at which irreversible freezing injury occurs. Ögren (1999) estimated critical temperatures by a 50% reduction in Fv/Fm ratio. Our method of estimating critical temperature is similar to that of Lindgren and Hallgren (1993), who suggested that critical temperature corresponded to a fixed Fv/Fm. Since reduction in Fv/Fm is linear following freezing lower temperatures, a critical temperature corresponding to 50% Fv/Fm reduction or 20% cambium visual damage was estimated by a linear model for each provenance. The correlation coefficient between critical temperatures estimated by chlorophyll fluorescence and cambium visual scoring damage was higher ($r = 0.73$) than those based on direct comparisons between % Fv/Fm reduction and visual cambium damage (r ranged from 0.57 to 0.70).

We conclude that chlorophyll fluorescence can be used as a simple method to rank cold hardiness among aspen provenances. Significant genetic variation among 37 provenances was indicated by the chlorophyll fluorescence technique. However, due to different responses to freezing by different tissues, variance expressed among provenances detected by CF and CVS was not completely consistent.

CONCLUSION

An effective method to rank genetic entities according to breeding targets, such as cold hardiness, possesses high economic value in tree breeding programs. The high correlation coefficients between stem Fv/Fm, and CVS or EC suggest that stem Fv/Fm may be a preferred indicator of cold hardiness development and provenance ranking due to its simplicity, nature and less time required for the process. However, the relative efficiencies of using these methods to detect genetic and adaptive variation were not completely consistent. The intraclass correlation coefficient estimated by CF and CVS were similar, but significantly lower than those estimated by electrical conductivity, suggesting that EC is superior over CF and CVS for detecting genetic variation. Any of these methods can be a good indicator of adaptive variation in cold hardiness of aspen populations in September, but the adaptive mechanism detected by each method might be different. In October, only EC method could detect adaptive variation among aspen population in cold hardiness. Thus, stem Fv/Fm may be good to rank provenances, but relative poor to detect genetic or adaptive variation among provenances.

The cold hardiness development of northwestern provenances could be divided into two phases. The first phase occurred at early-middle September and the second phase occurred at later September. The cold hardiness developed very fast during the second phase. Provenances from different parts of studying show different acclimate time and rate, which resulted in a poor correlation in cold hardiness in September and October. Thus, only ranking provenances in one occasion might be inaccurate and result in wrong conclusions.

Significant provenance effects in this study suggest that aspen is a highly differentiated species in this trait. Very high variance components expressed among provenances from northwestern Ontario, suggesting rapid gain could be obtain through provenance selection. Intraclass correlation coefficients were slightly greater in the first phase than in the second phase. Furthermore, variation among provenances follows noticeably the operational environmental factors in both phases. Growing season length and precipitation in August at the site of each seed origin were the best predicators of the second phase, while temperatures were always the best predicators of the first phase. Thus provenance performance can be significantly predicted by climatic variables that vary a great deal over fairly short geographic distance in northwestern Ontario, and this provides special information for aspen seed transfer.

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Appendix A

Average RC and Fv/Fm ratio for each provenance for 1999 experimental data for part I

		EC_Stem								CF_Leaf			
		KRKM				C45				KRKM		C45	
		5-Sep		5-Oct		15-Sep		6-Oct		5-Sep		15-Oct	
PROV	T*	MEAN	SE	MEAN	SE	MEAN	SE	MEAN	SE	MEAN	SE	MEAN	SE
1	1	0.336	0.010	0.205	0.006	0.252	0.002	0.209	0.007	0.827	0.007	0.810	0.008
2	1	0.304	0.011	0.210	0.006	0.233	0.008	0.208	0.008	0.837	0.002	0.815	0.005
3	1	0.334	0.003	0.232	0.005	0.259	0.002	0.217	0.013	0.835	0.008	0.827	0.004
8	1	0.284	0.009	0.244	0.009	0.253	0.005	0.201	0.010	0.825	0.015	0.827	0.006
9	1	0.319	0.002	0.261	0.024	0.231	0.020	0.248	0.009	0.825	0.005	0.810	0.013
10	1	0.266	0.010	0.258	0.002	0.237	0.002	0.216	0.001	0.822	0.011	0.807	0.008
17	1	0.290	0.012	0.268	0.001	0.250	0.009	0.227	0.007	0.812	0.006	0.815	0.011
18	1	0.296	0.011	0.266	0.003	0.248	0.009	0.205	0.007	0.825	0.006	0.827	0.004
19	1	0.278	0.016	0.233	0.001	0.240	0.004	0.220	0.020	0.822	0.011	0.825	0.002
23	1	0.304	0.009	0.288	0.006	0.242	0.005	0.241	0.017	0.817	0.010	0.820	0.004
25	1	0.318	0.014	0.217	0.014	0.234	0.010	0.195	0.001	0.822	0.007	0.825	0.002
26	1	0.306	0.008	0.239	0.021	0.247	0.009	0.222	0.005	0.825	0.008	0.797	0.016
1	2	0.525	0.011	0.245	0.016	0.487	0.009	0.331	0.011	0.387	0.224	0.730	0.005
2	2	0.451	0.003	0.267	0.004	0.453	0.015	0.321	0.012	0.65	0.054	0.662	0.022
3	2	0.521	0.006	0.275	0.013	0.437	0.009	0.329	0.013	0.622	0.082	0.672	0.033
8	2	0.470	0.021	0.269	0.009	0.468	0.012	0.314	0.004	0.810	0.009	0.675	0.029
9	2	0.517	0.003	0.304	0.004	0.432	0.021	0.307	0.012	0.805	0.006	0.735	0.027
10	2	0.430	0.007	0.261	0.001	0.442	0.008	0.320	0.018	0.777	0.026	0.720	0.015
17	2	0.427	0.008	0.268	0.002	0.513	0.001	0.307	0.011	0.780	0.004	0.557	0.117
18	2	0.541	0.003	0.267	0.002	0.498	0.008	0.345	0.004	0.757	0.018	0.712	0.020
19	2	0.600	0.007	0.259	0.003	.	.	0.268	0.005	0.790	0.021	0.722	0.026
23	2	0.527	0.015	0.292	0.020	0.430	0.009	0.267	0.012	0.712	0.061	0.605	0.058
25	2	0.503	0.012	0.234	0.031	0.531	0.002	0.281	0.033	0.782	0.016	0.677	0.028
26	2	0.503	0.004	0.241	0.003	0.497	0.006	0.261	0.009	0.702	0.031	0.725	0.029
1	3	0.714	0.020	0.322	0.002	0.655	0.011	0.404	0.014	0.140	0.082	0.432	0.031
2	3	0.645	0.015	0.342	0.003	0.633	0.017	0.360	0.007	0.400	0.036	0.367	0.037
3	3	0.619	0.012	0.345	0.009	0.540	0.008	0.343	0.010	0.442	0.049	0.417	0.027
8	3	0.595	0.003	0.367	0.014	0.652	0.005	0.358	0.005	0.215	0.074	0.332	0.029
9	3	0.676	0.015	0.369	0.005	0.548	0.004	0.367	0.014	0.665	0.013	0.462	0.035
10	3	0.611	0.005	0.328	0.011	0.538	0.008	0.381	0.008	0.322	0.029	0.425	0.022
17	3	0.611	0.015	0.314	0.004	0.646	0.002	0.338	0.008	0.357	0.148	0.385	0.006
18	3	0.665	0.014	0.320	0.005	0.592	0.007	0.409	0.007	0.377	0.028	0.417	0.030
19	3	0.727	0.009	0.353	0.007	0.516	0.009	0.343	0.012	0.280	0.096	0.395	0.016
23	3	0.615	0.006	0.355	0.005	0.570	0.010	0.345	0.004	0.337	0.016	0.372	0.028
25	3	0.66	0.040	0.303	0.008	0.633	0.012	0.339	0.015	0.362	0.037	0.337	0.039
26	3	0.635	0.032	0.327	0.007	0.602	0.014	0.334	0.006	0.462	0.057	0.402	0.024

* For September tests, the '1', '2' and '3' represents 5°C, –10°C and –20°C; For October tests, '1', '2' and '3' represents 5°C, –20°C and–40°C respectively.

Appendix B

Average Fv/Fm ratio, visual scoring damage and corresponding standard error for each provenance of 2000 experimental data for part I

		CF_Stem				VS**				CF_Leaf**	
		15-Sep		1-Oct		15-Sep		1-Oct		1-Oct	
PROV	T*	MEAN	SE	MEAN	SE	MEAN	SE	MEAN	STDRR1	MEAN	SE
1	1	0.608	0.017	0.567	0.008					0.751	0.011
2	1	0.617	0.027	0.500	0.031					0.754	0.018
3	1	0.536	0.014	0.539	0.016					0.764	0.011
8	1	0.602	0.016	0.501	0.026					0.766	0.008
9	1	0.600	0.026	0.495	0.012					0.773	0.001
10	1	0.587	0.038	0.530	0.027					0.769	0.017
17	1	0.537	0.018	0.513	0.030					0.769	0.007
18	1	0.510	0.039	0.522	0.018					0.740	0.021
19	1	0.578	0.026	0.549	0.037					0.736	0.011
23	1	0.549	0.011	0.470	0.013					0.753	0.007
25	1	0.563	0.037	0.503	0.027					0.737	0.002
26	1	0.534	0.020	0.558	0.027					0.768	0.007
1	2	0.518	0.021	0.497	0.048	15.2	4.02	0.0	0.0	0.636	0.002
2	2	0.478	0.021	0.434	0.063	40.4	9.96	0.0	0.0	0.623	0.025
3	2	0.409	0.015	0.496	0.021	60.0	19.22	0.0	0.0	0.680	0.013
8	2	0.451	0.020	0.466	0.029	60.8	4.31	0.0	0.0	0.631	0.015
9	2	0.479	0.011	0.445	0.011	56.0	17.23	0.0	0.0	0.670	0.012
10	2	0.476	0.024	0.493	0.030	19.6	9.26	0.0	0.0	0.652	0.014
17	2	0.440	0.013	0.447	0.024	0.0	0.00	0.0	0.0	0.691	0.005
18	2	0.405	0.013	0.459	0.019	53.6	6.85	2.0	2.0	0.646	0.002
19	2	0.470	0.013	0.486	0.030	43.6	11.95	5.2	3.2	0.668	0.027
23	2	0.401	0.016	0.439	0.024	34.4	4.70	0.0	0.0	0.634	0.016
25	2	0.445	0.013	0.477	0.013	7.2	7.20	0.0	0.0	0.702	0.008
26	2	0.469	0.008	0.494	0.034	22.8	19.48	0.0	0.0	0.674	0.015
1	3	0.315	0.004	0.413	0.019	48.4	11.82	8.8	3.61		
2	3	0.310	0.013	0.399	0.006	60.8	10.61	19.6	5.34		
3	3	0.320	0.014	0.415	0.011	65.2	11.79	12.4	3.86		
8	3	0.341	0.003	0.389	0.011	64.4	6.01	12.8	4.07		
9	3	0.320	0.015	0.418	0.019	81.2	4.22	5.8	0.58		
10	3	0.364	0.012	0.406	0.012	42.5	18.48	19.6	6.5		
17	3	0.368	0.014	0.397	0.005	59.6	12.15	15.8	2.45		
18	3	0.274	0.014	0.400	0.015	68.0	7.56	16.8	2.93		
19	3	0.338	0.014	0.409	0.011	70.8	7.76	5.6	1.69		
23	3	0.364	0.023	0.406	0.016	47.2	11.56	5.6	1.43		
25	3	0.401	0.030	0.402	0.010	63.6	10.55	14.8	2.24		
26	3	0.433	0.025	0.410	0.007	52.0	15.72	12.0	4.42		

Appendix B

Average Fv/Fm ratio, visual scoring damage and corresponding standard error for each provenance of 2000 experimental data for part I
(Continued)

		CF_Stem				VS**				CF_Leaf***	
		15-Sep		1-Oct		15-Sep		1-Oct		1-Oct	
PROV	T*	MEAN	SE	MEAN	SE	MEAN	SE	MEAN	STDRR1	MEAN	SE
1	4	0.213	0.015	0.361	0.006	50.8	15.57	23.6	2.63	0.369	0.031
2	4	0.233	0.009	0.338	0.004	71.2	11.77	44.8	2.41	0.326	0.022
3	4	0.23	0.015	0.345	0.016	82.0	13.49	14.0	5.62	0.415	0.037
8	4	0.186	0.017	0.317	0.017	100.0	0.00	36.0	11.09	0.323	0.032
9	4	0.254	0.018	0.330	0.021	48.4	5.81	22.8	6.88	0.358	0.013
10	4	0.224	0.010	0.331	0.007	57.6	17.56	31.6	2.03	0.304	0.028
17	4	0.328	0.045	0.306	0.003	66.0	12.69	46.0	2.52	0.448	0.040
18	4	0.190	0.005	0.323	0.012	67.2	9.72	42.0	1.26	0.344	0.030
19	4	0.259	0.015	0.365	0.015	64.8	6.82	22.4	7.30	0.396	0.023
23	4	0.310	0.014	0.343	0.021	52.0	12.45	16.4	6.07	0.388	0.029
25	4	0.258	0.029	0.336	0.021	50.4	10.62	18.4	2.56	0.426	0.017
26	4	0.301	0.035	0.375	0.004	62.8	9.62	14.8	2.57	0.411	0.021

*For September tests, '1', '2', '3' and '4' represents 5°C, -10°C, -15°C and –20°C respectively; For October tests, '1', '2', '3' and '4' represents 5°C, -10°C, -22°C and –33°C.
**Visual scoring was only tested at temperature '2', '3' and '4'.
***Leaf Fv/Fm ratios were only measured at temperature '1', '2' and '4'.

Appendix C

Average I_t (index of injury) for each provenance in part II, sorted by sampling date, site and test temperature

	September, 1999						October, 1999						November, 1999	
	KRKM		C45		DR		KRKM		C45		DR		KRKM	
P\T(°C)	-10	-20	-10	-20	-10	-20	-20	-40	-20	-40	-20	-40	-30	-50
1	28.46	56.97	31.40	53.92	3.58	18.60	4.96	14.67	15.39	24.71	7.19	17.45	4.83	6.32
2	21.13	48.96	28.68	52.11	5.67	18.95	7.28	16.77	14.28	19.23	4.96	12.37	3.12	5.88
3	28.10	42.83	24.07	37.91	3.28	23.60	5.59	14.73	14.27	16.13	4.04	15.61	5.80	6.85
4	27.81	46.28	40.59	53.14	4.26	25.97	13.56	23.02	10.24	18.13	6.65	16.04	6.53	8.09
5	24.90	44.82	34.94	49.25	5.64	27.22	5.86	13.98	13.02	17.03	2.68	13.18	.	.
7	23.55	39.11	36.26	40.61	2.07	15.61	0.38	13.34	10.62	22.07	1.98	10.05	0.72	4.91
8	26.03	43.50	28.83	53.42	8.06	22.10	3.33	16.24	14.13	19.67	10.24	15.78	3.76	4.70
9	29.17	52.47	26.16	41.23	1.98	17.46	5.82	14.66	7.90	15.84	2.65	13.61	4.26	4.58
10	22.39	47.02	26.79	39.49	4.00	18.26	0.51	9.48	13.27	21.05	4.99	12.79	6.23	7.04
12	31.17	45.91	31.95	40.92	3.23	17.24	4.13	13.49	4.46	17.94	1.04	9.18	1.71	2.15
14	28.45	50.60	32.99	36.64	5.62	21.50	11.52	20.37	13.08	20.33	.	.	0.76	1.73
15	30.71	41.23	27.79	44.21	1.26	14.46	0.25	6.90	9.31	14.27	6.31	12.22	3.15	4.13
17	19.21	45.15	35.06	52.75	5.36	19.99	0.01	6.41	10.24	14.35	.	.	1.08	2.46
18	34.86	52.43	33.24	45.76	6.90	28.27	5.16	15.37	17.64	25.70	8.62	16.97	4.82	13.82
19	44.59	62.19	.	55.38	7.35	21.39	3.36	15.57	6.05	15.78	5.97	15.73	3.25	7.01
20	27.64	55.92	35.69	50.43	6.37	18.41	-1.93	10.94	0.92	14.13	5.12	11.20	0.08	1.55
23	32.13	44.68	24.81	43.35	8.54	28.28	0.52	9.415	3.39	13.70	6.58	13.63	2.86	4.71
24	30.83	60.90	30.62	47.56	7.42	26.66	0.43	13.98	11.71	20.22	7.33	14.64	2.31	5.15
25	27.13	50.21	38.87	52.15	6.25	18.21	2.25	11.04	10.62	17.83	5.44	16.05	1.61	1.82
26	28.48	47.40	33.24	47.21	4.47	18.75	0.32	11.63	4.94	14.35	4.09	9.93	0.25	0.45

Appendix D

PC scores for each provenance from principal components analysis in part II

Provenance	PC1	PC2	PC3	PC4
1	1.083	-0.02974	0.60653	-1.2424
2	0.31958	-0.50098	1.10538	-0.52225
3	0.30817	-1.41614	-1.23956	0.75309
4	1.49293	-0.45888	1.64662	2.25424
5.	.	.	.	
7	-1.01025	-1.2264	0.91994	-0.84601
8	0.86681	0.62215	0.1157	-0.69305
9	-0.38701	-0.92421	-0.28174	0.85255
10	-0.09961	-1.23756	-0.80436	-1.53567
12	-1.19502	-0.48505	0.23189	1.10129
14.	.	.	.	
15	-0.98211	-0.53125	-0.89478	-0.42157
17.	.	.	.	
18	1.92732	0.25025	-1.01334	-0.16953
19.	.	.	.	
20	-1.13915	1.69629	0.62448	0.00354
23	-0.31244	1.19499	-1.94868	1.0864
24	0.55888	1.41384	-0.52084	-0.37888
25	-0.15402	0.95407	1.02739	-0.61151
26	-1.27709	0.67861	0.42539	0.36977

Note: Provenance #5, #14, #17 and #19 were only tested at one test temperature, thus the scores for these provenances could not be determined.